THE

BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

JOHN M. ANDERSON, Cornell University
ARTHUR L. COLWIN, Queens College, New York
DONALD P. COSTELLO, University of North Carolina
FRANK M. FISHER, JR., Rice University
CATHERINE HENLEY, University of North Carolina
MEREDITH L. JONES, Smithsonian Institution
ROBERT K. JOSEPHSON, Case Western Reserve University
CHARLES B. METZ, University of Miami
LEONARD NELSON, Medical College of Ohio at Toledo
HOWARD A. SCHNEIDERMAN, University of California, Irvine
STEPHEN A. WAINWRIGHT, Duke University
CARROLL M. WILLIAMS, Harvard University

W. D. RUSSELL-HUNTER, Syracuse University
Managing Editor

VOLUME 138

JANUARY TO JUNE, 1970

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $5.00. Subscription per volume (three issues), $14.00.

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between May 23 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

Second-class postage paid at Lancaster, Pa.

LANCASTER PRESS, INC., LANCASTER, PA.

MARINE BI
LIBRARY
RATORY
MASS.

CONTENTS

No. 1. February, 1970

Ahmed, Muzammil and Albert K. Sparks — Page
Chromosome number, structure and autosomal polymorphism in the marine mussels *Mytilus edulis* and *Mytilus californianus* 1
Ferguson, John C.
An autoradiographic study of the translocation and utilization of amino acids by starfish 14
Fingerman, Milton
Dual control of the leucophores in the prawn, *Palaemonetes vulgaris*, by pigment-dispersing and pigment-concentrating substances 26
Manzi, John J.
Combined effects of salinity and temperature on the feeding, reproductive, and survival rates of *Eupleura caudata* (Say) and *Urosalpinx cinerea* (Say) (Prosobranchia: Muricidae) 35
Postlethwait, John H. and Howard A. Schneiderman
Induction of metamorphosis by ecdysone analogues: *Drosophila* imaginal discs cultured *in vivo* 47
Sastry, A. N.
Reproductive physiological variation in latitudinally separated populations of the bay scallop, *Aequipecten irradians* Lamarck 56
Stunkard, Horace W.
The marine cercariae of the Woods Hole Massachusetts region 66
Vowinckel, C.
The role of illumination and temperature in the control of sexual reproduction in the planarian *Dugesia tigrina* (Girard) 77
Wiese, Lutz and David W. Shoemaker
On sexual agglutination and mating-type substances (Gamones) in isogamous heterothallic chlamydomonads. II. The effect of concavavalin A upon the mating-type reaction 88
Wilson, Wilfred J.
Osmoregulatory capabilities in isopods: *Ligia occidentalis* and *Ligia pallasii* 96

No. 2. April, 1970

Calder, Dale R.
Hydroid and young medusa stages of *Dipurena strangulata* (Hydrozoa, Corynidae) 109
Cherbas, Lucy and Peter Cherbas
Distribution and metabolism of α-ecdysone in pupae of the silkworm *Antheraea polyphemus* 115
Chiu, K. W. and W. G. Lynn
The effect of ACTH on the sloughing frequency of normal and thyroidectomized *Anolis carolinensis* (Iguanidae, Lacertilia) 129
Gooch, James L. and Thomas J. M. Schopf
Population genetics of marine species of the phylum Ectoprocta 138
Robertson, James D.
Osmotic and ionic regulation in the horseshoe crab *Limulus polyphemus* (Linnaeus) 157
Rustad, Ronald C., Shuhei Yuyama and Lynne C. Rustad
Nuclear-cytoplasmic relations in the mitosis of sea urchin eggs. II. The division times of whole eggs and haploid and diploid half-eggs 184

SLAUGHTER, MARGARET, FLORENCE C. ROSE AND ANTHONY LIUZZI PAGE

The effect of cesium-137 gamma rays on regeneration in *Tubularia* 194

STEINBACH, A. B.

Diurnal movements and discharge characteristics of electric gymnotid fishes in the Rio Negro, Brazil 200

TAYLOR, PETER B.

Observations on the function of the frontolateral horns and horn glands of barnacle nauplii (Cirripedia) 211

THOMAS, MARY BETH

Transitions between helical and protofibrillar configurations in doublet and singlet microtubules in spermatozoa of *Stylochus zebra* (Turbellaria, Polycladida) 219

WALKER, JOANNE G.

Oxygen poisoning in the annelid *Tubifex tubifex*. I. Response to oxygen exposure 235

No. 3. JUNE, 1970

COLE, K. S. AND D. L. GILBERT

Jet propulsion of squid 245

DIETZ, THOMAS H. AND RONALD H. ALVARADO

Osmotic and ionic regulation in *Lumbricus terrestris* L. 247

FENAUX, LUCIENNE

Maturation of the gonads and seasonal cycle of the planktonic larvae of the Ophiuroid *Amphiura chiajei* Forbes 262

HEATH, JAMES EDWARD AND ROBERT K. JOSEPHSON

Body temperature and singing in the katydid, *Neoconocephalus robustus* (Orthoptera, Tettigoniidae) 272

HOLLAND, NICHOLAS D. AND MICHAEL T. GHISELIN

A comparative study of gut mucous cells in thirty-seven species of the class Echinoidea (Echinodermata) 286

LEONE, DONALD E.

The maturation of *Hydroides dianthus* 306

MANGUM, DOROTHEA CASKEY

Burrowing behavior of the sea anemone *Phyllactis* 316

OHTAKI, TETSUYA AND CARROLL M. WILLIAMS

Inactivation of α-ecdysone and cyasterone by larvae of the fleshfly, *Sarcophaga peregrina*, and pupae of the silkworm, *Samia cynthia* 326

RICE, JOHN R., CONSTANTIN PAPASTEPHANOU AND DAVID G. ANDERSON

Isolation, localization and biosynthesis of crassin acetate in *Pseudoplexaura porosa* (Houttuyn) 334

ROSE, S. MERYL

Restoration of regenerative ability in ligated stems of *Tubularia* in an electric field 344

SMITH, MICHAEL J.

The blood cells and tunic of the ascidian *Halocynthia aurantium* (Pallas). I. Hematology, tunic morphology, and partition of cells between blood and tunic 354

SMITH, MICHAEL J.

The blood cells and tunic of the ascidian *Halocynthia aurantium* (Pallas). II. Histochemistry of the blood cells and tunic 379

SUMMERS, WILLIAM C. AND JOHN J. MCMAHON

Survival of unfed squid, *Loligo pealei*, in an aquarium 389

Vol. 138, No. 1 February, 1970

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

CHROMOSOME NUMBER, STRUCTURE AND AUTOSOMAL POLYMORPHISM IN THE MARINE MUSSELS *MYTILUS EDULIS* AND *MYTILUS CALIFORNIANUS*[1]

MUZAMMIL AHMED[2] AND ALBERT K. SPARKS

College of Fisheries, University of Washington, Seattle, Washington 98105

The bay mussel *Mytilus edulis* (Linnaeus) and the California sea mussel *Mytilus californianus* (Conrad), are polymorphic in color, shape and size. They are found intertidally on rocks, stones, or pilings and sometimes in deeper water down to 10 to 20 or 25 fathoms (Soot-Ryen, 1955). *Mytilus edulis* is found in several parts of the world and has been considered by some as a cosmopolite, although this opinion has been debated by Soot-Ryen (1955). *M. edulis* is native to the Pacific coast of North America, but may occur sympatrically with *M. californianus* which is common along the more exposed beaches in crashing waves and, indeed, occurs only where there is surf (Ricketts, Calvin, and Hedgpeth, 1968). Along the exposed beaches, the presence of *M. edulis* appears to be related to the presence of fresh water streams, and at such places individuals of both species may occupy the same rocks. The two species are easy to distinguish except that very small specimens may be difficult to separate from one another.

Mytilus edulis is normally dioecious but rare hermaphroditic individuals can be found (Sagiura, 1962). While reviewing reproduction in molluscs, Fretter and Graham (1964) indicated that individuals of *M. californianus* emitting sperms or eggs are true males and females and that there are no immature gametes of the opposite sex in the gonad. Both species broadcast their gametes and fertilization occurs externally.

On the West coast of the United States, *M. edulis* commences spawning in late April or early May and continues until late August. Young (1942) indicated that in *M. californianus*, beginning in September, spawning gradually increases to reach a maximum in mid-winter and then declines to a minimum from May to August. Occasionally they will spawn in summer, but usually only to a limited extent.

Both species have served as popular research material for embryological and physiological studies. However, nothing is known of their comparative cytogenetics. It is not known whether the forms of the two species are ecophenotypes or

---

[1] Contribution No. 319 from the College of Fisheries, University of Washington, Seattle, Washington 98105.

[2] Permanent Address: Department of Zoology, University of Karachi, Karachi, Pakistan.

Copyright © 1970, by the Marine Biological Laboratory
Library of Congress Card No. A38-518

are genetically different. However, Schlieper (1957) indicated that *M. edulis* populations in the region of the North Sea (30‰) and Western Baltic (15‰) did not belong to genetically or physiologically distinct races since physiological differences disappeared when specimens from the two populations were transposed.

A chromosome study of the two species from several intertidal locations along the Pacific coast of the United States was, therefore, initiated to elucidate the cytogenetic structure.

Material and Methods

Mussels were obtained from several rocky intertidal beaches in Puget Sound and the Pacific coastline of the United States. All observations of chromosome morphology were made from unfertilized eggs (primary oocytes), fertilized eggs, and 2 to 6 cell embryos and morulae. Eggs needed for intraspecific crosses were obtained either by the induction of artificial spawning or by stripping the ripe gonads. When sexually mature, both species spawned after rough handling, shaking in a bucket, by changing of water or by thermal shock. Spawning behavior of the two species is, however, very much unpredictable.

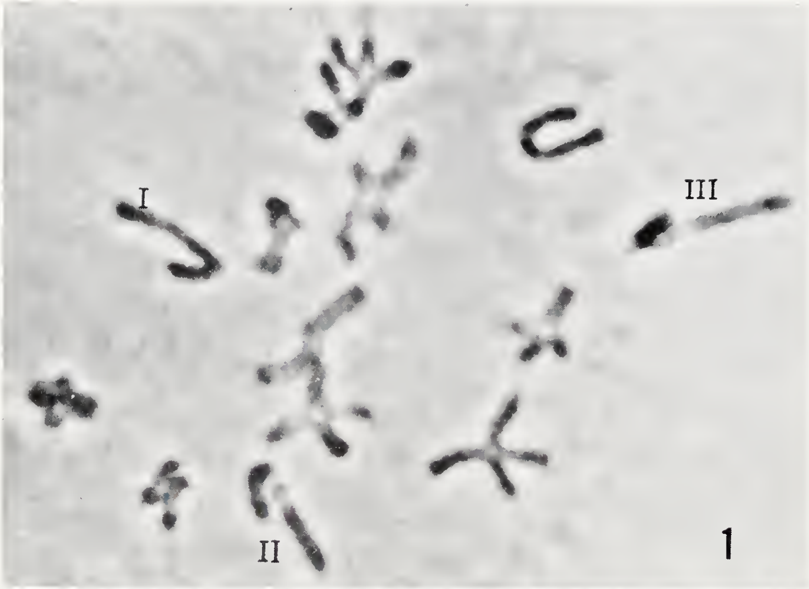
A single *M. edulis* can produce eggs in the number of 10^7 (Fretter and Graham, 1964) and *M. californianus*, probably as many or more. The ripe eggs of the two species obtained by spawning or stripping the gonads measure approximately 60 μ in diameter and are somewhat opaque in *M. edulis* and orange colored in *M. californianus*.

Intra-specific crosses were made by adding a few drops of very dilute sperm suspension to thousands of eggs contained in 500 ml of filtered sea water in a beaker. Fertilization and cleavage occurred at room temperature. Samples of eggs and embryos were fixed and preserved in 1:3 acetic alcohol (Glacial acetic acid and absolute ethyl alcohol). All chromosome observations were made from temporary aceto-orcein stained squash preparations of eggs and embryos. Squashes were made as follows: several embryos were placed on a slide and air dried; stained in

Table I

Number of mussels examined from different locations

	Location	Number of animals examined
Mytilus edulis		
State of Washington	Golden Gardens (Seattle)	3
	Alki Point (Seattle)	2
	Bainbridge Island	3
	Coupville	3
State of California	Humboldt Bay	5
Mytilus californianus		
State of Washington	Agate Beach	4
	Clallum Bay	1
	Cattle Point (Friday Harbor)	1
	Port Townsend	2
	Cape Flattery	3
State of Oregon	Cannon Beach	3
State of California	Morro Bay	5



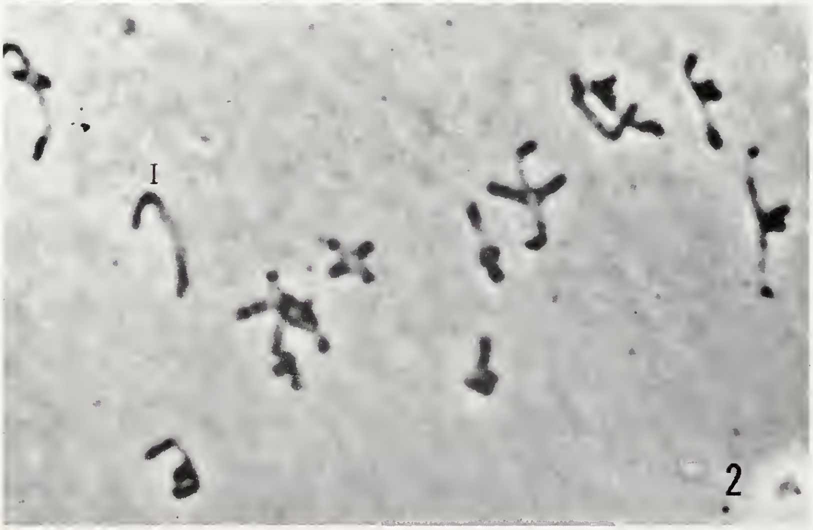


FIGURE 1. Polar view of fourteen metaphase I bivalents of *Mytilus edulis* from Golden Gardens; I, II and III heteromorphic bivalents, × 1512, phase contrast.

FIGURE 2. Equatorial view of fourteen metaphase I bivalents of *Mytilus californianus* from Agate Beach; I heteromorphic bivalent, × 1512, phase contrast.

a drop or two of aceto-orcein for two to several minutes; squashed under a coverslip; extra stain was blotted off and the slide was heated on a flame for a few seconds. This could be examined immediately or sealed off by applying a mixture of lanolin and paraffin wax and kept for later use.

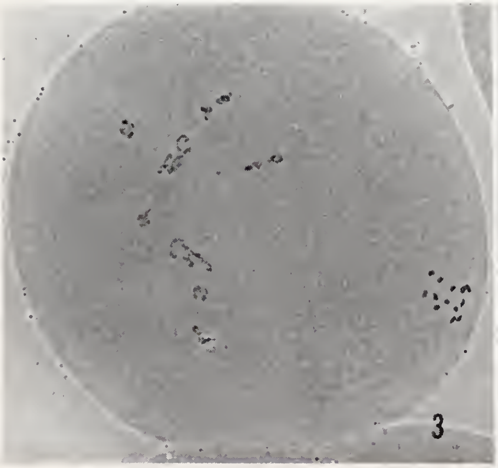
Chromosome plates were examined and photographed on a Zeiss Photomicroscope employing a green or blue green filter. Camera lucida drawings of chromosomes were made on an American Optical scope using 15 × oculars and 97 × objectives. Extreme care was exercised while making camera lucida drawings so that the proportion of chromosome arms and absolute lengths were not distorted.

Observations

The number of mussels examined cytologically from the respective collection sites is shown in Table I. Suitable material of *M. edulis* was available from Golden Gardens in Seattle, Washington, and Humboldt Bay in California, and for *M. californianus* from Agate Beach and Cape Flattery in the State of Washington. In the material from other localities, counts of meiotic bivalents could be made but a detailed study of their morphology was not possible due to their extreme condensation, crowding and some degree of stickiness.

Meiosis and mitosis

In the two mussels, a natural blocking of meiosis occurs at prometaphase or metaphase I. In the spawned eggs (primary oocytes), the meiotic bivalents were usually observed at prometaphase to metaphase I (Figs. 1 and 2), although diplotene-diakinesis stages were seen also, in evidently immature eggs. Meiosis is completed after the reactivation of the eggs by a fertilizing sperm. The pronucleus of the sperm could often be seen in the egg cytoplasm and sometimes the chromosomes of the male genome were clearly observed (Fig. 3). After fertilization, maturation divisions followed, polar bodies were extruded (Fig. 4) and cleavage occurred. Stages of mitosis were easily seen in cleaving eggs.



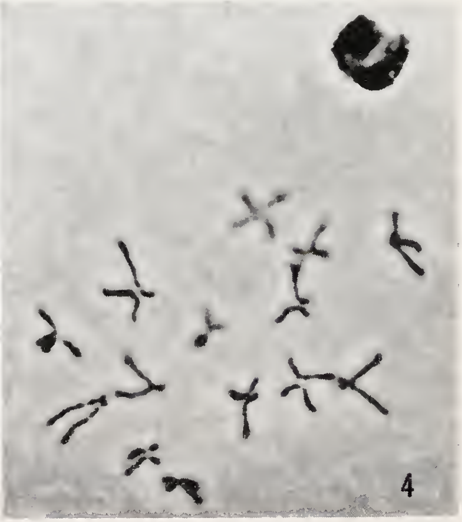


FIGURE 3. A fertilized oocyte of *M. edulis* showing metaphase I bivalents in the center and 14 chromosomes of the male genome at 4 o'clock position. Two chromosomes may appear as one due to close proximity, × 480, phase contrast.

FIGURE 4. Fourteen metaphase II chromosomes and polar body extrusion in *M. edulis*. × 1200, phase contrast.

Size and counts of chromosomes

During the meiotic prometaphase the bivalents of the two mussels showed some degree of stretching. The largest bivalent of *M. edulis* stretched in some cases to 11 μ and that of *M. californianus* to 15 μ. At metaphase I the bivalents of the two species measured approximately 8 to 3 μ. The mitotic chromosomes, at mid-metaphase, measured approximately 10 to 3 μ in 4 to 6 cell stage embryos. Both meiotic and mitotic chromosomes showed a slight decrease in size from the largest to the smallest, which made their individual identifications difficult.

In hundreds of eggs examined from both mussels, a constant count of 14 bivalents was made (n = 14) and is, therefore, consistent for 16 specimens of *M. edulis* and 19 specimens of *M. californianus* from different localities (Table I). Fourteen haploid chromosomes of the male genome were seen in fertilized but uncleaved eggs (Fig. 3). The reduced number of 14 chromosomes could be observed at all other stages of maturation divisions and especially at metaphase II of female meiosis (Fig. 4).

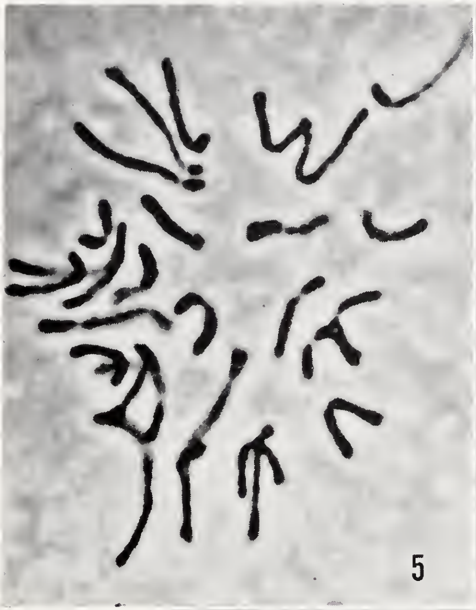
Mitotic chromosome counts were made from *M. edulis* material from Golden Gardens and Humboldt Bay and of *M. californianus* from Agate Beach and Cape Flattery. Twenty eight chromosomes were generally counted (Figs. 5 and 6) in mitotic plates of both species (2n = 28) but hyperdiploid sets of 29 or 30 and such atypical sets as 27, 26, 25, 24 and others also occurred. A mitotic plate of *M. edulis* with 26 chromosomes is shown in Figure 7. In one 5-celled embryo of this species, one nucleus was at interphase but the other four contained 19 to 20 metaphase chromosomes. In *M. californianus,* aneuploid counts of similar nature were made. Approximately 5–10% of the nuclei contained atypical plates. In both species haploid metaphases with 14 chromosomes were also observed and polyploid sets were also present in less than 1% of the examined mitotic plates.

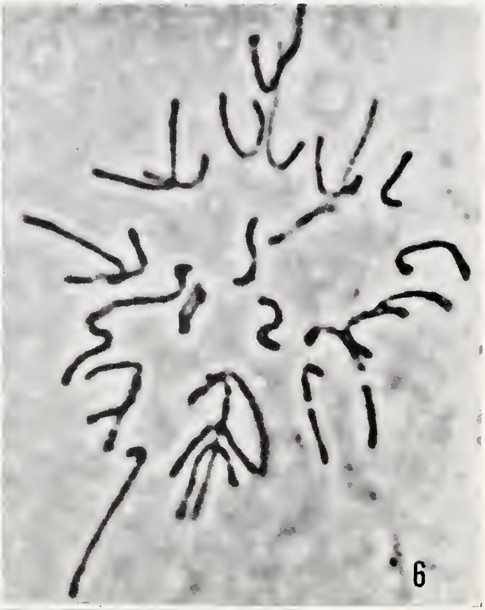
Chromosome terminology

The following terminology was used in the characterization of chromosome shapes: metacentric (M) two armed, and the relative lengths of the arms approach unity; submetacentric (S) unequal armed, J-shaped chromosome; acrocentric (A) one very small and another very large arm, the centromere is subterminal and interstitial; telocentric (T) one armed, the centromere being strictly terminal. The definitions of metacentric, acrocentric, and telocentric chromosome are borrowed, after slight rewording, from John and Lewis (1968). The distinction between metacentric, submetacentric, and acrocentric chromosome is clearly a relative one. Truly equal armed metacentrics were few, so that slightly unequal armed V-shaped chromosomes were also included in this category. Chromosomes in which the ratio of the small to large arm was approximately 1:2 were considered submetacentric.

Chromosome morphology

No differential heteropycnosity was observed in any of the bivalents or mitotic chromosomes at any observable stage of meiosis or mitosis, so, on this basis, the chromosomes are all autosomal.





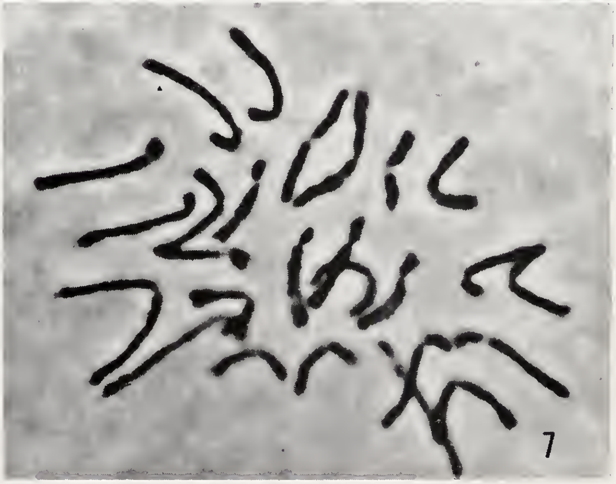
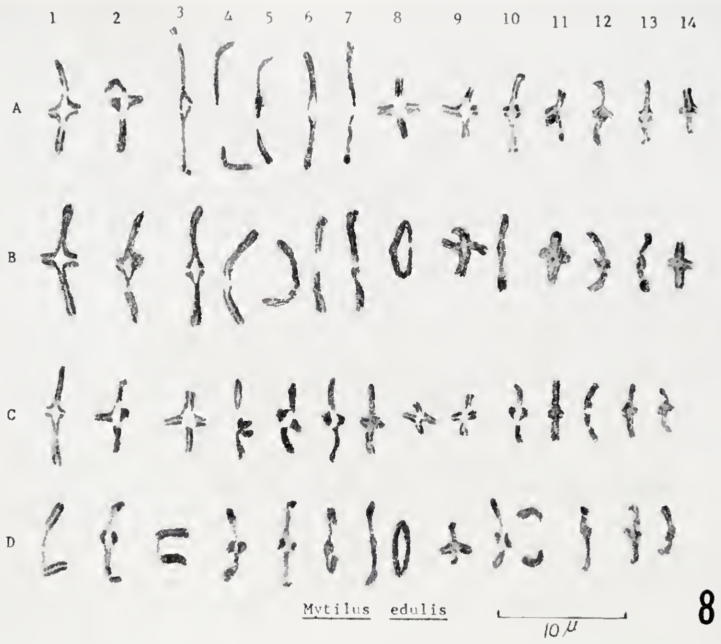


FIGURE 5. Polar view of 28 mitotic metaphase chromosomes of *M. edulis,* × 1512, phase contrast.

FIGURE 6. Polar view of 28 mitotic metaphase chromosomes of *M. californianus,* × 1512, phase contrast.

FIGURE 7. Aneuploid mitotic plate of *M. edulis* with 26 chromosomes, × 1920, phase contrast.

Camera lucida drawings of the meiotic bivalents of *M. edulis* from Humboldt Bay and *M. californianus* from Cape Flattery are shown in Figures 8 and 9, respectively. In these, bivalents were arranged according to the diminishing order of their lengths and the assignment of positions is arbitrary. In the standard meiotic complements of the two mussels (row C, Figs. 8 and 9) six to seven



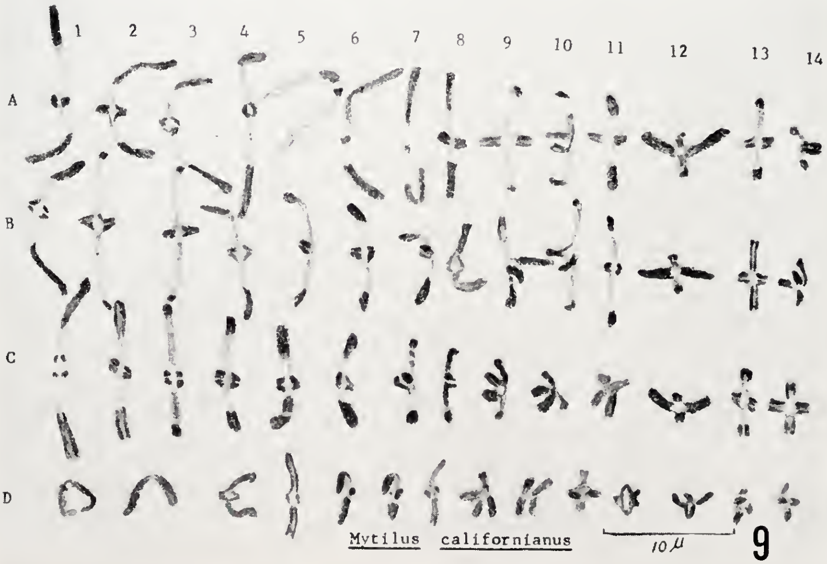


FIGURE 8. Camera lucida drawings of prometaphase and metaphase I bivalents of a single specimen of *M. edulis* from Humboldt Bay.

FIGURE 9. Camera lucida drawings of prometaphase and metaphase I bivalents of a single specimen of *M. californianus* from Cape Flattery.

bivalents resembled crosses of equal or unequal arms and possessed interstitial chiasmata (see also Figs. 1 and 2) and three to four bivalents were rod like. Each of the 14 bivalents of the two mussels possessed, generally, a single chiasma at prometaphase or metaphase I. Based on the analysis of the female metaphase I bivalents the chiasma frequency in the two mussels amounts to 1.0 and the recombination index 28. This was, however, exceeded in some plates due to the presence of a single ring bivalent which was observed in approximately 5% of the examined plates of *M. edulis* and even fewer plates of *M. californianus.*

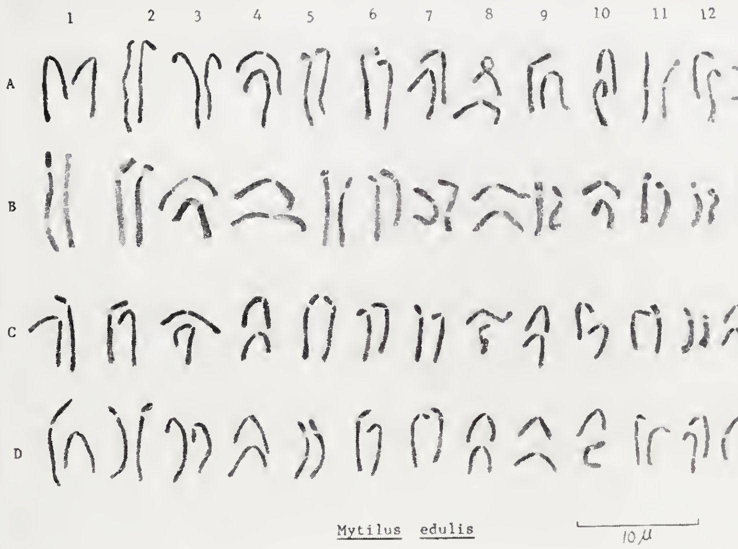
Morphology of mitotic chromosomes was critically examined in camera lucida drawings of 10 complements of *M. edulis* and 8 of *M. californianus,* although numerous other plates were observed for this purpose. The centromeric positions of the chromosomes were clearly seen so that their characterization as metacentric, submetacentric, and acrocentric was relatively easy. In both species, usually three pairs of chromosomes were acrocentric and the rest metacentric or submetacentric. Telocentric chromosomes were either absent or only an occasional one was observed. Secondary constrictions were generally not seen, although they may be present; for instance on chromosome 1 in row A (Fig. 11).

Chromosome polymorphism

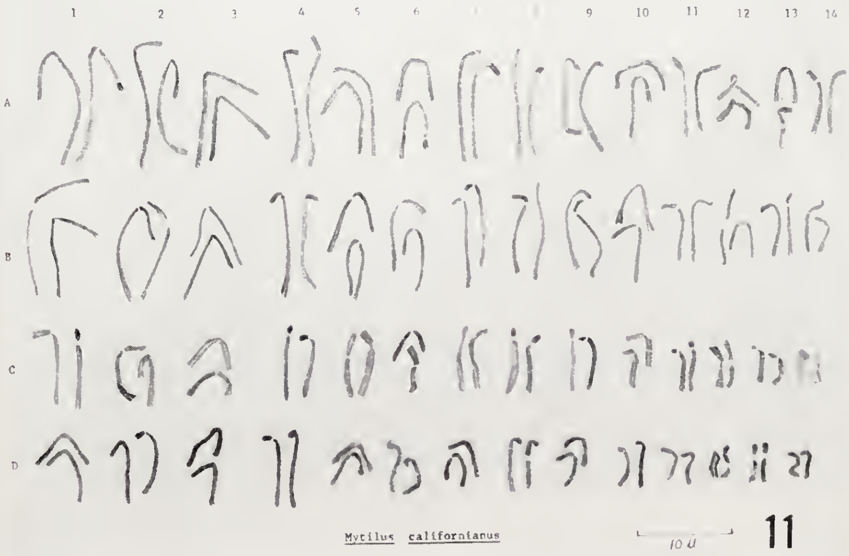
Structural chromosome polymorphism in both meiotic and mitotic chromosomes was observed and was attributed to the presence of pericentric inversions or centric-shifts (centromere shifts) in autosomes.

In approximately 5% of the meiotic plates of both mussels, along with cross and rod like bivalents, J-shaped or inverted J-shaped bivalents were observed or an occasional asymmetrical bivalent was seen. These were clearly heteromorphic bivalents, that is, the centromeres of the chromosomes composing them existed at different positions. For example, the three bivalents of *M. edulis* marked I, II, and III (Fig. 1) are heteromorphic and so is bivalent I of *M. californianus* (Fig. 2). Similarly, bivalent 4 of *M. edulis* (row A, Fig. 8) and bivalent 6 and 7 of *M. californianus* (row A, Fig. 9) are also heteromorphic. These latter two bivalents of *M. californianus* are composed of metacentric and acrocentric chromosomes and resemble the bivalents heterozygous for "elastic constrictions" reported by White (1957) in morabine grasshoppers. The frequency of such bivalents remains to be determined but they did not appear to be present in more than 5% of the meiotic plates examined from a single specimen of *M. californianus* from Cape Flattery. Meiotic complements shown in row A of Figures 8 and 9 are inversion or centric-shift heterozygotes, since they contain hetermorphic bivalents.

Another aspect of polymorphism in meiotic bivalents involved the substitution of metacentric or ring bivalents in place of acrocentric or telocentric rod bivalents and vice versa. In *M. edulis,* bivalent 8 (row B and D, Fig. 8) is a ring bivalent and may be an alternate form of the acrocentric bivalents at the same position in row A and C of this figure. Bivalent 2 and 3 of *M. californianus* (row B, Fig. 9) are acrocentric bivalents, but bivalents 2 and 3 in row A of the same figure are composed of metacentric chromosomes. Bivalent 11 of this species (row D, Fig. 9) is a ring bivalent although others at this position in rows A to C are rod or cross like bivalents.



10



11

FIGURE 10. Karyotypes of *M. edulis* from Golden Gardens based on camera lucida drawings of 4 complements of a single specimen.

FIGURE 11. Karyotypes of *M. californianus* from Cape Flattery (row A and B) and Agate Beach (row C and D) based on camera lucida drawings of 2 complements of 1 specimen each from the two localities.

A distinct autosomal polymorphism was observed in mitotic karyotypes also. Camera lucida drawings of 4 mitotic complements of a single specimen of Golden Gardens *M. edulis* are shown in Figure 10 and of one specimen each of *M. californianus* from Cape Flattery (row A and B) and from Agate Beach (row C and D) in Figure 11. In these sets, homologues of the 14 chromosome pairs were established on the basis of centromere positions and chromosome lengths. In some cases metacentric and acrocentric (MA), metacentric and submetacentric (MS), submetacentric and acrocentric (SA), and telocentric and acrocentric (TA) chromosomes had to be placed together so that heteromorphic pairs resulted. Examples of heteromorphic homologues present in Figures 10 and 11 will be mentioned below:

In Figure 10, pair 1 in row B and C of *M. edulis* is clearly heteromorphic, being composed of acrocentric and metacentric (AM) and metacentric and acrocentric (MA) chromosomes respectively; pair 2 in row B is composed of submeta- and acrocentric (SA) elements and pair 14 in this row, of submetacentric and metacentric (SM) elements. In Figure 11, pair 8 in row A and B of *M. californianus* is composed of telocentric and acrocentric (TA) and submetacentric and telocentric (ST) elements; pair 1 in row C and D is heteromorphic, being composed of submetacentric and acrocentric (SA) and metacentric and submetacentric (MS) elements, respectively; pair 2 in row D is heteromorphic for submetacentric and acrocentric (SA) elements.

Along with heteromorphic chromosome pairs MS, MA and SA *etc.*, metacentric (MM), submetacentric (SS), and acrocentric (AA) pairs seemed to replace each other at the same positions in different complements of the two species. This may be due to pericentric inversions or centric-shifts in both chromosomes of a pair. In *M. edulis*, the arrangement of chromosome pairs in rows A to D in Figure 10 on position 7 is: MM, MM, AA and AA; on 9: MM, SS, MM, MM; on 11: MM, AA, SS, and AA; and on 12: SS, AA, AA, and SS, respectively. In *M. californianus* the arrangement in rows A to D in Figure 11 on position 7: SS, AA, AA, and MM; on 9: SA, SS, AA, and MM; on 10: MM, MM, MM, and AA; on 11: SS, SS, AA, and MM; and on 13: MM, SS, AA, and SS, respectively. Slight variation of centromeric positions in the corresponding arms of some other homologues were present but will not be emphasized here. These may be due to small inversions, duplication deficiencies or merely differential contraction of arms.

The occurrence of hetermorphic chromosome pairs and pairs carrying homozygous inversions or centric-shifts introduces a variation in the number of different kinds of chromosomes in mitotic complements. In both mussels, 2 to 6 pairs of acrocentric chromosomes were present in different complements. In the face of so much variation in chromosome morphology, it was difficult to denote any one complement as standard. The four complements of the single specimen of *M. edulis* from Golden Gardens shown in Figure 10 differ from each other morphologically to a slight or greater extent. This is also true for the four sets belonging to two specimens of *M. californianus* (Fig. 11) from Cape Flattery and Agate Beach. Complement D of *M. edulis* (Fig. 10) is a completely homozygous set, whether inversion or standard. Complement B of *M. californianus* (Fig. 11) would have been homozygous except for a heteromorphic pair on position 8.

DISCUSSION

This paper confirms the chromosome counts of 2n = 28 (n = 14) reported by us earlier (Ahmed and Sparks, 1967b) for both *M. edulis* and *M. californianus*. Menzel's recent paper (1968) assigns the number 2n = 24 (n = 12) to *M. edulis* from the East coast of the United States. It is possible that intraspecific chromosome number variations might exist between the East and West coast forms of *M. edulis,* yet there were no numerical variations in our counts of meiotic bivalents from the hundreds of eggs from each of the 16 specimens of *M. edulis* and 19 specimens of *M. californianus*. Some atypical counts were made during mitosis and 2n = 24 in our material was considered aneuploid and existed in only a few cases.

Aneuploid counts ranging from 19 to 27 and of 29 or 30 chromosomes were made in approximately 5–10% of the mitotic plates examined. These might have been due to any or all of such processes as non-disjunction or abnormal segregation, centric fusions and centric fissions. The exact cause or causes of aneuploidy in both species remain to be determined but some decreased counts in mitotic plates might be due to centric fusions. A centric fusion involves the fusion of two non-homologous acrocentrics or rod like chromosomes to form a metacentric or V-shaped chromosome, thereby decreasing the chromosome number. White (1954) emphasized that centric fusions will occur in animal species with more than one pair of acrocentric chromosomes. In the two mussels way is open for centric fusions to occur in the presence of 2 to 6 pairs of acrocentrics. Hyperdiploid counts of 29 or 30 might have been, also, due to the presence of supernumeraries.

The 14 meiotic bivalents of the two mussels were, with few exceptions, homomorphic, and isopynotic at all observable stages of meiosis. They were, therefore, all autosomal. None of the bivalents displayed any other characteristic of a sex-bivalent. There is no evidence for the existence of sex-chromosomes in any of the marine pelecypods which have been investigated cytologically. The bivalents of *Mercenaria mercenaria, M. campechiensis* and their hybrids (Menzel and Menzel, 1965), and those of the oysters *Ostrea lurida* and *Crassostrea gigas* (Ahmed and Sparks, 1967a) and *Crassostrea virginica* (Longwell, Stiles and Smith, 1967) are all homomorphic and isopycnotic. The sex-determining mechanism of *C. virginica,* the American oyster, may be along the lines of the multifactorial hypothesis or a system of polygenes (Montalenti and Bacci, 1951). Here, various combinations of 4 pairs of genes responsible for sex-determination may produce long or short, or equally long male and female phases. True males and females occur when crossing over and recombination gives rise to more extreme forms. This latter condition might prevail in *M. edulis* and *M. californianus* since both species reproduce, generally, as true males and females and sex-reversal usually does not occur.

Evidence of structural rearrangements exists in both mussels. Heteromorphic bivalents were observed in less than 5% of meiotic plates of both species. One or two such bivalents of *M. californianus* resembled the bivalents heterozygous for "elastic constrictions" reported by White (1957) in the grasshopper, *Keyacris (Moraba) scurra* populations. Such bivalents were considered by White to constitute a "minute" structural change.

The presence of heteromorphic pairs in meiosis as well as mitosis, the substitution of rod bivalents in place of metacentric or ring bivalents and vice versa, indicates that pericentric inversions or centric-shifts are involved. A centric-shift affecting one of a pair of chromosomes may give rise to a heteromorphic bivalent in meiosis and heteromorphic homologues in mitosis. A homologous centric-shift may change a rod shaped acrocentric or telocentric bivalent into a metacentric or ring shaped bivalent. Pericentric inversions or centric-shifts in autosomes are known to occur in several animal species and the pertinent literature has recently been reviewed by John and Lewis (1968).

The presence of heterozygous pericentric inversions or centric-shifts in homologous autosomes will ordinarily lead, due to free crossing over, to the production of genetically unbalanced gametes. The mussels must have evolved a mechanism to escape the consequences of such a phenomenon. Both mussels, however, could support a large wastage of gametes since a vast number of them is produced. *M. edulis* and *californianus* are very successful species and the autosomal polymorphism does not seem to have hindered their fitness and, indeed, it may even be of adaptive significance. The visible polymorphism (polyphenism) in mussel populations may be due to floating genetic differences. The population cytology of the two mussels needs further investigation in the light of this preliminary evidence of autosomal polymorphism.

We wish to express our gratitude to Dr. H. L. Roman, Chairman of the Department of Genetics and Dr. W. S. Hsu of the Department of Zoology of the University of Washington for their help in the interpretation of some observations. Thanks are due to Dr. D. W. Hagen, of the College of Fisheries, for comments and criticism of the manuscript and for several suggestions and discussions.

Summary

The bay mussel *Mytilus edulis* (Linnaeus) and the California sea mussel *Mytilus californianus* (Conrad) have a common diploid chromosome number 28 ($n = 14$) that is, there were 14 bivalents during meiosis and 28 chromosomes during mitosis. A total of 35 specimens of the two species were examined from several intertidal locations in Puget Sound and the Northwest Pacific coast of the United States. The number of meiotic bivalents in stripped or spawned eggs was always constant but aneuploid counts of mitotic chromosomes were made in cleaving eggs and embryos. Heteromorphic bivalents in meiotic plates and heteromorphic homologues in mitotic karyotypes were observed. The number of metacentric, submetacentric and acrocentric chromosomes was variable. There were 2 to 6 pairs of acrocentrics in different complements. This polymorphism indicates the existence of pericentric inversions or centric-shifts in both meiotic and mitotic chromosomes.

LITERATURE CITED

Ahmed, M., and A. K. Sparks, 1967a. A preliminary study of chromosomes of two species of oysters (*Ostrea lurida* and *Crassostrea gigas*). *J. Fish. Res. Board Can.*, **24**: 2155–2159.

AHMED, M., AND A. K. SPARKS, 1967b. Chromosomes of oysters, clams and mussels. *Proc. Nat. Shellfish. Ass.*, **58**: 10.

FRETTER, V., AND A. GRAHAM, 1964. Reproduction, pp. 127–164, *In:* K. M. Wilbur and C. M. Yonge, Ed., *Physiology of Mollusca, Volume 1.* Academic Press, New York.

JOHN, B., AND K. R. LEWIS, 1968. The chromosome complement. *Protoplasmatol. Handb. Protoplasmaforsch.*, **6A**: 1–206. Springer-Verlag. Wien, New York.

LONGWELL, A. C., S. S. STILES AND D. G. SMITH, 1967. Chromosome complement of the American oyster *Crassostrea virginica* as seen in meiotic and cleaving eggs. *Can. J. Genet. Cytol.*, **9**: 845–856.

MENZEL, R. W., 1968. Chromosome number in nine families of pelecypod mollusks. *Nautilus*, **82**: 45–50, 53–58.

MENZEL, R. W., AND M. Y. MENZEL, 1965. Chromosomes of two species of quahog clams and their hybrids. *Biol. Bull.*, **129**: 181–188.

MONTALENTI, G., AND G. BACCI, 1951. Osservazioni e ipotesi sulla determinazione del sesso negli ermafrodite. *Sci. Genet. (Torino)*, **4**: 5–12.

RICKETTS, E. F., J. CALVIN AND J. W. HEDGPETH, 1968. *Between Pacific Tides.* [4th Ed.] Stanford University Press, Stanford, California. 614 pp.

SAGIURA, Y., 1962. Electrical induction of spawning in two marine invertebrates (*Urechis unicinctus*, hermaphrodite *Mytilus edulis*). *Biol. Bull.*, **123**: 203–206.

SCHLIEPER, C., 1957. Comparative study of *Asterias rubens* and *Mytilus edulis* from North Sea (30‰) and the Western Baltic Sea (15‰). *Annee Biol.*, **33** (3–4): 117–127.

SOOT-RYEN, 1955. A report on the family Mytilidae (Pelecypoda). *Allan Hancock Pac. Exped.*, **26** (1): 1–174.

WHITE, M. J. D., 1954. *Animal Cytology and Evolution.* University Press, Cambridge, 454 pp.

WHITE, M. J. D., 1957. Cytogenetics of the grasshopper *Moraba scurra.* IV. Heterozygosity for "Elastic Constrictions." *Aust. J. Zool.*, **5**: 348–354.

YOUNG, R. T., 1942. Spawning season of the California mussel, *Mytilus californianus. Ecology*, **23**: 490–492.

Reference: *Biol. Bull.*, **138**: 14–25. (February, 1970)

AN AUTORADIOGRAPHIC STUDY OF THE TRANSLOCATION AND UTILIZATION OF AMINO ACIDS BY STARFISH[1]

JOHN CARRUTHERS FERGUSON

Department of Biology, Florida Presbyterian College, St. Petersburg, Florida 33733

In a series of previous reports (Ferguson, 1964ab, 1966, 1968a) experimental evidence has been presented which indicates that the circulation of fluid in the coelomic spaces, together with active cellular mechanisms of uptake and exchange, are largely responsible for the translocation of nutritive substances throughout the bodies of starfish. While such studies have provided considerable insight into these physiological processes, a great deal is still unknown about the functioning of the distributive systems in these animals. In particular, little direct evidence has been obtained on the movements of nutrients through the thick layers of connective tissue comprising the body wall, or of the conductive functions of the complex of structures located in the ambulacral region—the water vascular system, the so-called "hemal system," and the perihemal canals. Furthermore, the primary source of nutrition of the epidermis is still in doubt, as this tissue has been shown to be capable of taking considerable subsistence directly from the exterior (Ferguson, 1967ab, 1968b).

While these particular anatomical regions do not lend themselves well to most kinds of experimental study, it was felt that some understanding of the nutritive physiology of these parts could be obtained by using the technique of autoradiography to examine the movements into and the retention by these regions of radioactivity from C^{14}-labeled amino acids previously delivered by injection into the interior of the animals. The following will report the results of such a study.

MATERIALS AND METHODS

Medium-size specimens of the starfish, *Echinaster echinophorus*, were collected from tidal grass flats near the southern shore of Tampa Bay. After being maintained in the laboratory for a few days, each was injected in the middle of the central disc with approximately 0.1 ml of filtered sea water containing 2–3 microcuries of C^{14}-labeled amino acid mixture (obtained from New England Nuclear Corporation of Boston, Massachusetts, and said to represent a synthetic *Chlorella* protein hydrolysate). In injecting the disc region, the bulk of the solution was delivered either into the lumen of the stomach or to the surrounding perivisceral coelom. While it was impossible to tell which at the time, the difference could easily be determined by the location of the radioactivity in the digestive glands demonstrated by the autoradiographs. Otherwise, the location of the injection seemed to make little difference in the results.

Immediately following the injection, the animals were rinsed several times in fresh sea water and then placed in tanks of sea water which was changed frequently, particularly during the first few hours. It was hoped that this procedure would prevent any of the labeled amino acid that might leak out of the injection injury from being taken up by the epidermis. Subsequent results verified the general success of this procedure, although in a few specimens some external uptake was detected. The animals were fed pieces of clam *ad libitum* while being retained.

---

[1] Supported by NSF grant GB 6906.

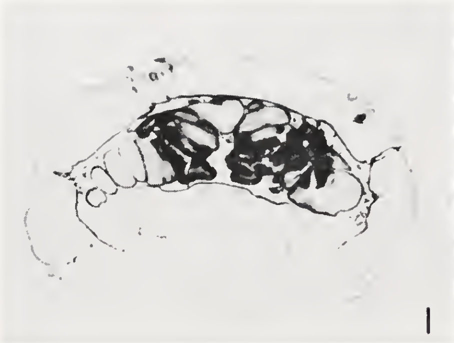
At intervals after the injection of 1 hour, 24 hours, 20 days, 35 days, 55 days and 75 days, pairs of animals were sacrificed and two of their arms removed. These were cut in half and placed in one-half strength Bouin's solution for several days (this not only fixed the tissues, but also served to decalcify them). The tissues were then dehydrated in alcohols and embedded in paraffin under vacuum. Sections were cut at 10 microns thickness and prepared as Kodak AR-10 stripping film autoradiographs. Groups of slides were developed and studied (unstained) after exposure periods ranging from 1 month to a year. The autoradiographs were compared to other sections stained in Mallory's phosphotungstic acid (PTA) hematoxylin.

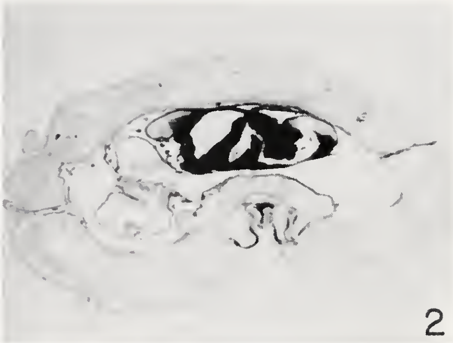
Observations

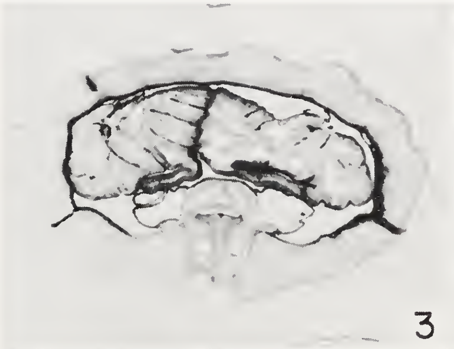
Much of the labeled amino acid administered in the disc was apparently rapidly translocated to various regions along the length of the arms and incorporated into insoluble material that could be visualized by the autoradiographic technique. Doubtless any remaining soluble compounds were lost during the processing of the sections or oxidized to CO_2 by the animals. Even in specimens sacrificed after just one hour, high retention of the tracer was observed in both the digestive glands and visceral peritoneum (Fig. 1). Particularly noteworthy was the uptake within the lining of the papulae extending through the body wall, and the area around the mesenteric supports. There was little difference in uptake between the proximal and the distal regions of the arms. Presumably the labeled amino acid was rapidly moved to all these places by ciliary currents maintained in the digestive glands and coelomic spaces. The existence of such currents have been documented in several species by Gemmill, 1915; Irving, 1924; Budington, 1942; Anderson, 1960; and others.

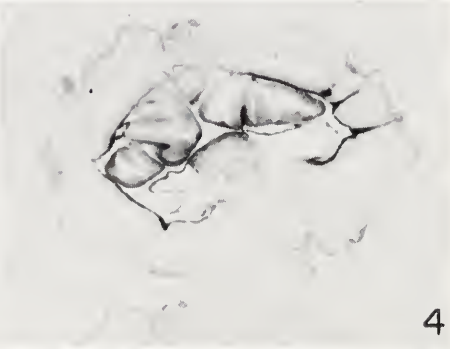
Amoebocytes located in all areas of the perivisceral coelom exhibited distinctive amounts of the label, although they generally did not take up and retain more of the C^{14}-amino acid than did the other cells exposed to the circulating fluids.

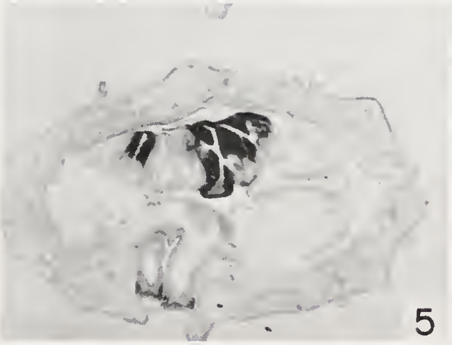
Much the same general distribution of labeled material as was found in the 1-hour specimens was also seen in those sacrificed at other time intervals (Figs. 2–6), along with a number of progressive changes. By 24 hours large quantities of retained labeled material had become located in the hemal system and associated connective tissue regions of the tube feet (Fig. 2) and by 5 days quantities of radioactive substances were seen dispersing in the connective tissue adjacent to the peritoneum and accumulating in a layer beneath the epidermis and in the outer regions of the large glands located in the dermis (Figs. 3, 7, and 8). Uptake by the lining cells of the tube feet was discernable at this time, probably indicating some translocation, *via* the water vascular fluid, from the very radioactive ampullar region. Labeled amoebocytes also began to appear in the body wall, water vascular system and various other places besides the perivisceral coelom. From 20 days on these dispersions continued with relative increases in radioactivity in most


1


2


3


4


5

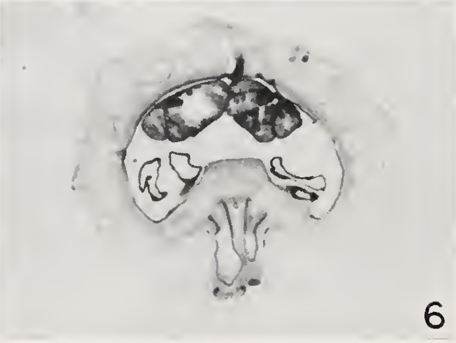

6

FIGURE 1. Unstained autoradiograph of arm section 1 hour after injection of C^{14}-amino acids. Note retention of radioactivity in tissues associated with perivisceral coelom. Slight darkening of epidermis is due to natural pigmentation and small amounts of tracer absorbed by this tissue directly from the exterior medium; 11 ×.

FIGURE 2. Section similar to that in Figure 1 from animal sacrificed 24 hours after injection. Note activity in hemal septum and connective tissue layer of tube feet; 10 ×.

FIGURE 3. Section similar to the previous ones from animal sacrificed 5 days after injection. Hemal septum is still radioactive. Some labeled amoebocytes are in the ampullae and the tube feet. Greater dispersion of radioactivity in the body wall is now discernable; 10 ×.

FIGURE 4. Section similar to the previous ones 20 days after injection. More dispersion of radioactivity through the dermis has occurred; 12 ×.

FIGURES 5 and 6. Sections similar to the previous ones from animals sacrificed at 55 and 75 days after injection. Much dispersion of radioactivity has occurred in the various tissues, but the most active areas are still the same as they were at 24 hours; 10 × and 14 ×.

areas of the body (Figs. 4, 5, and 6). These later changes were not dramatic, however, and most of the radioactivity remained in the same locations in which it was found at five days.

The 35-day, 55-day, and 75-day specimens seemed to require longer periods of exposure before satisfactory autoradiographs could be obtained, perhaps indicating metabolic utilization of the administered amino acid over the extended post-injection period.

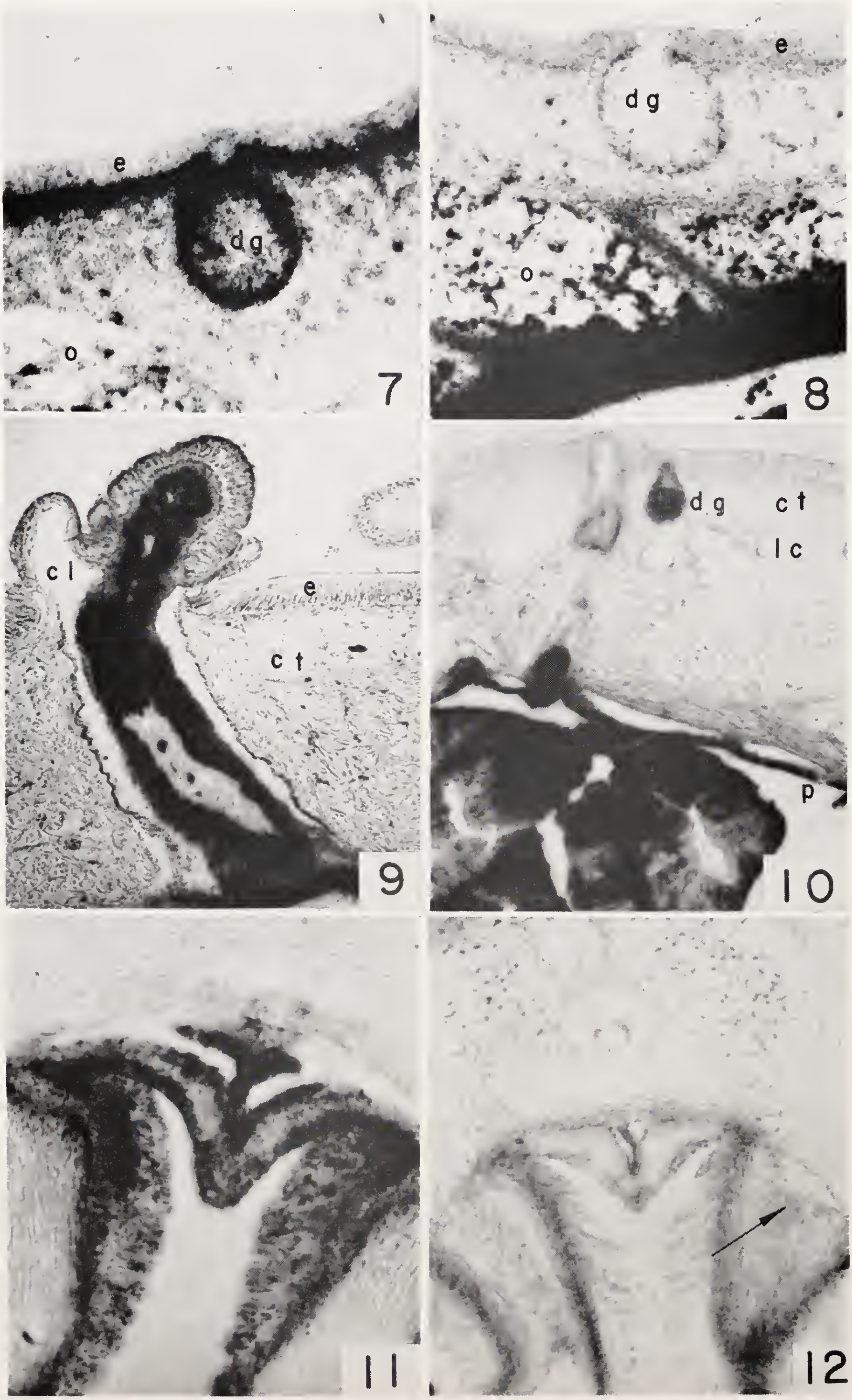
Intradermal translocation

While at 24 hours practically no labeled material was detected in the body wall, outside the peritoneum, significant translocation through the dermis was quite apparent in specimens sacrificed after 5 days (Figs. 7 and 8). In these animals radioactive material has moved from the peritoneum, through the fibrous meshwork of the connective tissue, all the way to the area beneath the epidermis. In Figure 8 the large quantities of radioactivity dispersing outward from the peritoneum are especially evident. The retention of the labeled material in the connective tissue area indicates that the administered C^{14}-amino acids have been synthesized into insoluble elements of the connective tissue, particularly mucopolysaccharide ground substance and collagenous fibers (see Ferguson, 1960). The retention of the labeled substance is greatest in the areas adjacent to the peritoneum and the epidermis. Study of stained sections indicate that both these areas contain numerous cells of various types and are probably prime locations for the production of connective tissue substance.

Uptake of tracer by individual cells could be detected freely throughout the dermal area in the 5-day specimens. There was an especially significant uptake by the osteocytes in all areas of the ossicles. As the solid nature of the ossicles would probably restrict the diffusion of the labeled nutrients into them, it seems likely that nutrients reach the interior of these structures by cell to cell transport. There was also a considerable uptake in the secretory cells of the large dermal glands, which was still evident even at 75 days (Fig. 10). This observation confirms that these organs obtain most of their raw materials from nutrient sources within the body.

While considerable radioactivity could be seen in the dermal region of most specimens after 5 days, practically no label was detected in their epidermis (Fig. 7 and others). As the great affinity of the epidermis for free exogenous nutrients has already been well documented (Ferguson, 1967b, 1968b), this observation strongly supports the hypothesis that there is some sort of functional diffusion barrier against amino acids, their products, and probably other nutrients located beneath the epidermis. Indeed, the distribution of radioactivity shown in Figure 7 remarkably complements that of previously published autoradiographs (Ferguson, 1967b) of animals exposed to C^{14}-labeled amino acids in their external media. Such a diffusion barrier would largely prevent losses of soluble nutrients from the animals, and require that the epidermis obtain the bulk of its nutrition from exogenous sources.

Running more or less randomly as a network throughout the outer regions of the body wall of most specimens are open lacunar channels (dermal spaces). One might expect these, together with the concentric lacunar spaces associated with



Figures 7–12.

the papulae (Fig. 9), to serve as channels for the dispersal of nutrients. In general, however, very little increased radioactivity could be detected in the vicinity of these areas in any of the sections. Thus, if they do have a nutrient transport function, it was not made evident by the limitations of the technique employed. Their general organization, however, would suggest a significant role in respiration.

Hemal system function

The appearance of large quantities of radioactivity in the hemal system and some of the adjacent lateral regions, beginning with the 24-hour specimens, was a startling observation and demanded careful study. A transport function was ascribed to this system by a number of workers of the last century and especially championed by Cuènot (1887). More recently, however, it has been widely believed that the system, in asteroids at least, is largely vestigial and that any transport of nutrients required by the ambulacral area would be accomplished in the adjacent perihemal spaces and water vascular system. An alternative function for the hemal system was reported by Unger (1962), who indicated that it transported neurosecretory products from the radial nerve cord to the water vascular system. He diagramed the hemal tissue as hollow vessels opening into the transverse water vascular canals.

As stated, the region of the hemal septum and its connecting structures became intensely radioactive beginning with the 24-hour specimens (Fig. 2), and they retained considerable activity even after 35 days (Fig. 11). After 55 and 75 days (Fig. 12), the intensity of the radioactivity was somewhat diminished, as it was throughout most regions of these animals.

Interestingly, the activity found in the hemal system and the lateral regions was not uniform along the length of the arms. Rather, it would build up over a number of serial sections and then decrease again to negligible amounts before beginning to build back up again. For this reason it is unlikely that the observed activity represented organic material transported centrifugally from the disc, as might be expected if the system were functioning as a simple vascular system.

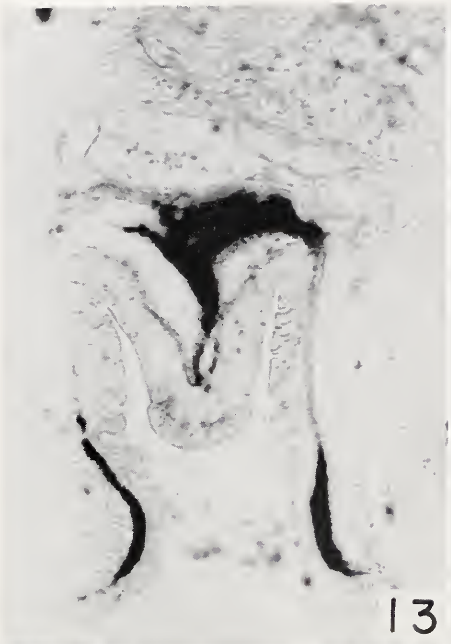
The radioactivity found in the hemal septum could be traced as being contiguous, *via* the transverse hemal processes, with large quantities of labeled substance found in sheets of connective tissue located in the middle layer of the tube

FIGURES 7 and 8. Autoradiographs of arm sections of animal 5 days after injection. Note how epidermis (e) remains almost free of the radioactivity found in the subepidermal region, and large dermal gland (dg). In addition to labeled material dispersing through the body wall, many radioactive cells may be seen including osteocytes (o) in the ossicles; 110 × and 85 ×.

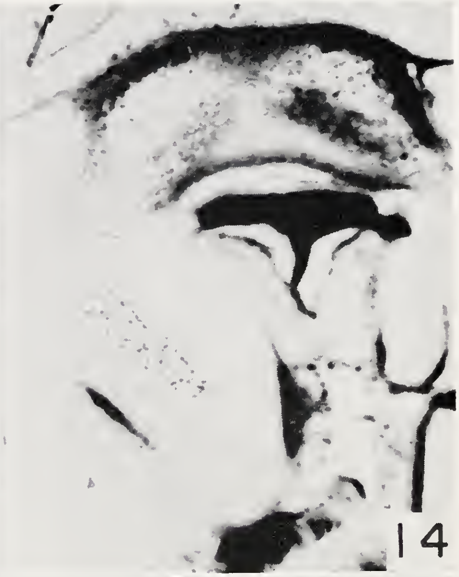
FIGURE 9. Autoradiograph of section through papula of specimen 24 hours after injection. While peritoneal lining of this structure is very radioactive, no activity has yet reached the body wall connective tissue (ct) or epidermis (e); (cl = circumferential lacuna); 100 ×.

FIGURE 10. Autoradiograph of section of aboral body wall from animal 75 days after injection. While radioactivity may be seen dispersed in the connective tissue (ct) and large dermal gland (dg), there is no significant increase in its level in the vicinity of the lacunar channels (lc); (p = peritoneum); 70 ×.

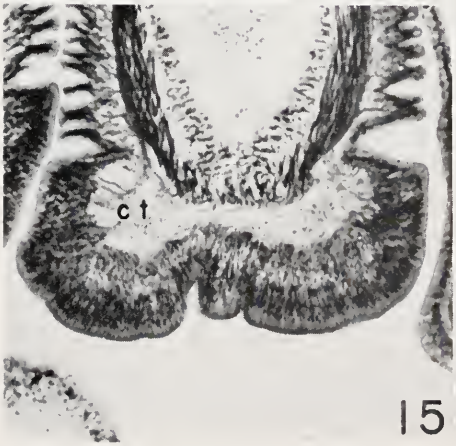
FIGURES 11 and 12. Autoradiographs of sections through ambulacral areas of animals sacrificed 35 and 75 days after injection. Some progressive dispersions of radioactivity from the hemal structures and tube foot connective tissue may be detected. In Figure 12 the tube foot lining cells (arrow) have apparently taken up radioactive material from the water canals; 110 × and 70 ×.



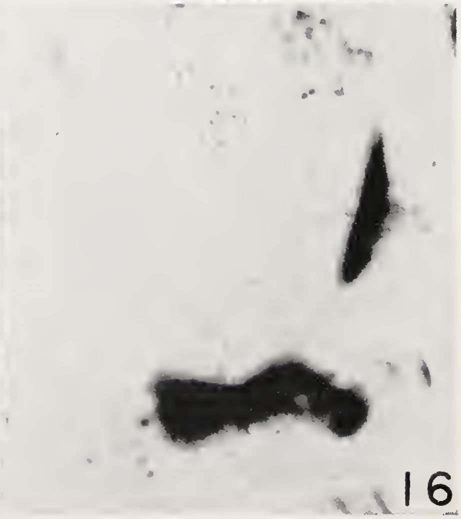
13



14



15



16

FIGURES 13 and 14. Autoradiographs of nearly adjacent sections through ambulacral area of animal sacrificed 24 hours after injection. Note the rather specific retention of the label in the hemal tissue and associated connective tissue layer of tube feet. Unlabeled amoebocytes are located in the bottom of left tube foot and radial water canal; 100 × and 70 ×.

FIGURE 15. Section of tube foot stained with PTA hematoxylin. Note how the connective tissue layer (ct) fans out into the sucker; 110 ×.

FIGURE 16. Autoradiograph of section similar to that in Figure 15 showing radioactive material in connective tissue layers 24 hours after injection; 100 ×.

foot walls (Figs. 13 and 14). This connective tissue is located between the longitudinal muscle cells and the elongate epidermal cells of these structures. Above the sucker, the connective tissue may be seen to "fan out" in stained sections (Fig. 15), and this area also collects radioactive material (Fig. 16). Some further dispersion of the activity amongst the epidermal cells of the sucker was observed in specimens kept for the longer periods of time (Figs. 5 and 6).

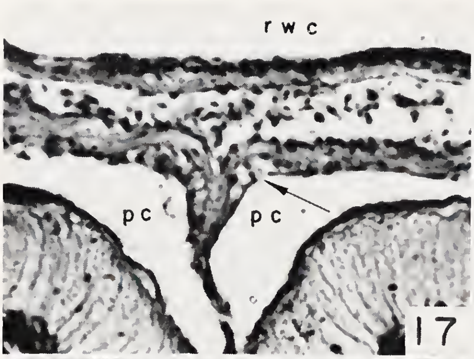
While in the 24-hour specimens some uptake of the tracer was seen occasionally in Lange's nerve and other areas exposed to the perihemal canals (Fig. 14), by far the major portion of the activity was found in the hemal structures and the connective tissue layers of the tube feet just described. In specimens retained for longer periods, slightly greater dispersion of activity into adjacent areas could be detected (Figs. 11 and 12).

The histological structure of the hemal system of these animals is very difficult to interpret. Study of stained sections (Figs. 17 and 18) indicate that it consists of a dense network of connective tissue and channels filled with numerous fixed cells, amoebocytes, and thick ground substance. In general appearance its structure is, on a smaller scale, not too dissimilar from that of the echinoid axial organ described by Millott (1966). As the ground substance stains intensely with the periodic acid-Schiff reaction (unaffected by salivary digestion), and with protein stains such as bromphenol blue, it is probably a mucopolysaccharide.

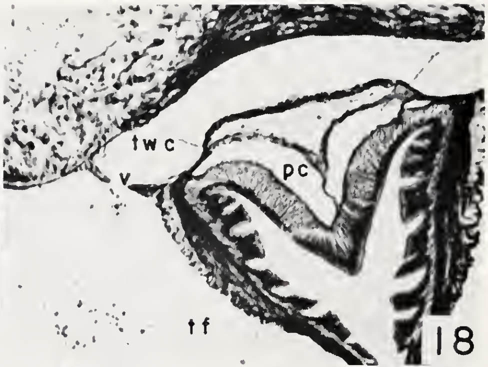
In the region in which the transverse water vascular canal passes out to connect with the tube foot (FIG. 18), the hemal septum also fans out and connects with the connective tissue of the wall of the transverse water vascular canal and sheets of connective tissue in the wall of the tube foot. In my sections there is no indication of a continuous hemal canal that might connect with this water vessel as described by Unger (1962). The spaces seen in the hemal tissue can usually be traced to openings into the perihemal canals.

A distinctive feature of the hemal system is the large number of amoebocytes usually found in and around it. They often fill up the hemal channels and particularly may be found in the perihemal space above the hemal septum where it fans out to make its lateral connections (Fig. 17). Possibly these cells could be responsible for bringing the labeled substance to the hemal tissue. In the 24-hour specimens labeled amoebocytes are found in the perihemal canals in the vicinity of the highly radioactive areas of the hemal septum. Few such cells, however, may be located elsewhere in the ambulacral area. In particular, numerous unlabeled amoebocytes are found in all areas of the water vascular system (Figs. 13, 14 and 16). While it is possible that amoebocytes could transport labeled amino acids in a soluble form which would not be preserved by the technique employed, other evidence, particularly the presence of heavily labeled amoebocytes elsewhere, would indicate that transport of such material by these cells is probably not significant.

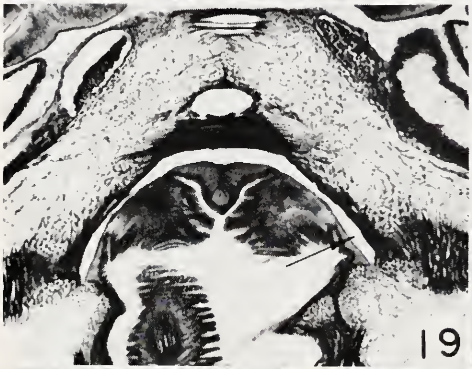
If the amoebocytes do not bring the bulk of the radioactivity to the hemal septum, where does this tissue obtain it? The probably answer may be found by tracing the lateral extensions of the perihemal canals in their circuit around the tube feet (Fig. 19). It is discovered that they make a rather lengthy juxtaposition with the perivisceral coelom (Fig. 20). Detailed study of this region also indicates that the peritoneal lining changes from its general columnar character



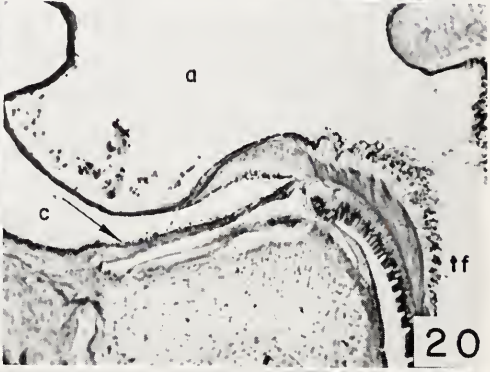
17



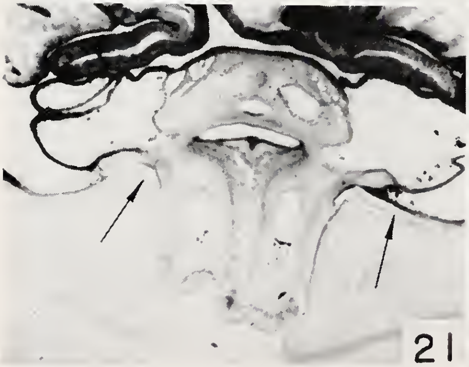
18



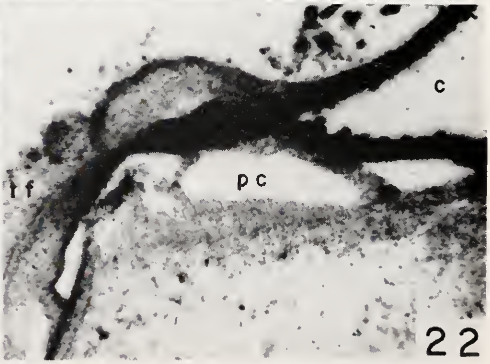
19



20



21



22

FIGURE 17. Section through hemal septum making its lateral connections; PTA hematoxylin. Note hemal channels (arrows) which open into perihemal canals (pc) and numerous amoebocytes, especially above hemal system; (rwc = radial water canal); 310 ×.

FIGURE 18. Section through ambulacral area showing lateral connections of the hemal septum with the wall of the transverse water canal (twc) and tube foot connective tissue layer; PTA hematoxylin (v = valve; tf = tube foot; pc = perihemal canal); 100 ×.

FIGURE 19. PTA hematoxylin stained section showing lateral extension of the perihemal canals (arrow); 40 ×.

FIGURE 20. PTA hematoxylin stained section showing part of the juxtaposition of the perihemal canal and the perivisceral coelom (c). The peritoneum becomes thinner in this region (arrow); (a = ampulla; tf = tube foot); 310 ×.

to a more cuboidal or squamous form. In some sections (Fig. 21 and 22) the whole region appears very radioactive.

It is, then, highly possible, that soluble nutritive substances are transported between the two cavities in these regions. They are circulated through the perihemal canals, and efficiently taken up by the hemal septum, particularly as the fluid percolates through its many canals. Here the soluble materials are reduced to an insoluble form (probably a mucopolysaccharide), which is transported in loose connective tissue sheets to the distal ends of the tube feet where it appears to be dispersed in the sucker region.

Discussion

While the present study represents an experimental situation, there is much reason to believe that it accurately reflects the normal functioning of the animals. Small quantities of a number of different amino acids may always be detected in the coelomic fluid of *Echinaster* (author's observation). The addition, then, of small amounts of these substances (in a labeled form) should make little biological difference. Indeed, previous work (Ferguson, 1964ab, 1968a) has demonstrated how such dissolved nutrients, in spite of their low concentrations, probably play a major role in the uniform distribution of nutritive materials throughout the body.

As a result of this study, it is now possible to formulate a much more complete concept of how cells in various isolated regions of the animals obtain their subsistence. The basic theory is as follows (subject to further modification as additional data is obtained): Nutritional substances are actively taken up from the environment by ingestion through the mouth into the digestive glands, and by direct absorption (particularly of free amino acids and sugars) into exposed epidermal cells. The material obtained by the latter is used exclusively by them and is not passed on to the more internal parts in appreciable quantities, probably because of the presence of a diffusion barrier (See Ferguson, 1967ab, 1968b). These epidermal cells may, however, release some material back into the environment.

The material taken into the digestive glands is digested, stored, and slowly released to the circulating coelomic fluid, which transports it to the major "core" regions of the body. All the cells (including those of the digestive glands) lining the coelomic cavity take up and release these products in an apparent exchange process.

Nutrients taken up by the somatic peritoneum are allowed to diffuse through the connective tissue network or possibly are converted to a viscous, insoluble ground substance which can do that. In either case, the translocation across the demal layer may be quite rapid, and active metabolic areas beneath the epidermis (including the large demal gland cells) do not suffer from nutritional lack. The

FIGURE 21. Autoradiograph of section from animal 5 days after injection. Note association of perihemal canals with perivisceral coelom (arrows); 25 ×.

FIGURE 22. More magnified view of right portion of Figure 21 to show association of perihemal canal (pc) and perivisceral coelom (c); (tf = tube foot); 310 ×.

nutritive material is largely blocked from entering the epidermis and leaving the body by the diffusion barrier located beneath this tissue.

Osteocytes, which form an interconnecting network throughout the ossicles, also are able to efficiently distribute nutrients amongst themselves, apparently by cell to cell transfer.

Cells located in the ampullae pass some of the nutritional products which they take up from the perivisceral coelom into the circulating fluid of the water vascular system. From here they may be taken up and utilized, particularly by the lining cells and muscle cells of the tube feet. Cells in certain specialized areas of the somatic peritoneum, lateral to the ampullae, similarly pass nutritive materials into the perihemal canals where they may be utilized by the cells lying adjacent to this compartment (particularly certain muscle and nervous elements). Much of the material appearing in the perihemal canals, however, is filtered out by the hemal structures and synthesized into insoluble compounds (apparently mucopolysaccharides) which are distributed through the connective tissue layer of the tube feet to the sucker region. Here they might be used as adhesive substances or for other purposes.

In their sojourns throughout the body, amoebocytes also pick up considerable quantities of nutritive materials. While these materials are generally retained in an insoluble form within the cytoplasm, the amoebocytes may, perhaps, disperse them in outlying regions, supplementing the other translocative systems.

Obviously, there is much more to be learned about the nutritional physiology of these animals. Little is yet known, for example, about the function of the axial organ and other hemal structures of the disc region. Application of techniques similar to those used in this study, however, should provide much useful insight into the function of these structures. Furthermore, the specificity with which the label was taken up by certain areas of the hemal system and tube feet in the present work suggests the possibility of biochemically identifying the substances being produced and transported by these regions using extraction and analytical procedures.

SUMMARY

1. Specimens of the starfish, *Echinaster echinophorus*, were injected in the disc region with small quantities of C^{14}-labeled amino acid mixture. After periods ranging from 1 hour to 75 days they were sacrificed and histological sections of their arms prepared as stripping film autoradiographs.

2. By 1 hour after the injection bound radioactive material was detected in most tissues in the proximity of the coelomic cavity, and while translocations from these areas were noted, most of the labeled material remained in these areas even after 75 days.

3. By 24 hours large quantities of radioactivity were found spasmodically in the hemal septum and traced dispersing outward into the connective tissue layer of the tube feet and the suckers. It is believed that this material was transported from the peri-visceral coelom *via* the perihemal canals and fixed as an insoluble mucopolysaccharide by the cells of the hemal septum.

4. By 5 days significant dispersion of radioactive material through all regions of the body wall was evident. Strong retention of the radioactive material was

found in the subepidermal layer, the large demal glands, osteocytes, and in the connective tissue layers adjacent to the somatic peritoneum.

5. As no sign of movement of radioactivity into the epidermis was seen in any of the sections, it is concluded that a previously theorized diffusion barrier beneath this layer must exist, and that epidermal cells obtain most of their nutrition from external sources.

6. Judging by their distribution in the various sections, it seems possible that the amoebocytes play a minor, purely secondary role in the dispersion of nutritive substances throughout the body.

LITERATURE CITED

Anderson, J. M., 1960. Histological studies on the digestive system of a starfish, *Henricia*, with notes on Tiedemann pouches in starfishes. *Biol. Bull.*, **119**: 371–398.

Budington, R. A., 1942. The ciliary transport-system of *Asterias forbesi*. *Biol. Bull.*, **83**: 438–450.

Cuénot, L., 1887. Contribution à l'étude anatomique des astérides. *Arch. Zool. Exp. Gen., Séries 2*, **5**: 1–144.

Ferguson, J. C., 1960. The nature of the connective tissue of the body wall, retractor harness and cardiac stomach of the starfish, *Asterias forbesi*. *Amer. Zool.*, **3**: 522.

Ferguson, J. C., 1964a. Nutrient transport in starfish. I. Properties of the coelomic fluid. *Biol. Bull.*, **126**: 33–35.

Ferguson, J. C., 1964b. Nutrient transport in starfish. II. Uptake of nutrients by isolated organs. *Biol. Bull.*, **126**: 391–406.

Ferguson, J. C., 1966. Insensitivity of starfish digestive glands to metabolic inhibitors and and drugs. *Amer. Zool.*, **6**: 529.

Ferguson, J. C., 1967a. Utilization of dissolved exogenous nutrients by the starfishes *Asterias forbesi* and *Henricia sanguinolenta*. *Biol. Bull.*, **132**: 161–173.

Ferguson, J. C., 1967b. An autoradiographic study of the utilization of free exogenous amino acids by starfishes. *Biol. Bull.*, **133**: 317–329.

Ferguson, J. C., 1968a. Transport of amino acids by starfish digestive glands. *Comp. Biochem. Physiol.*, **24**: 921–931.

Ferguson, J. C., 1968b. An autoradiographic analysis of the uptake of exogenous glucose by three species of starfishes. *Amer. Zool.*, **8**: 805.

Gemmill, J. F., 1915. On the ciliation of asterids, and on the question of ciliary nutrition in certain species. *Proc. Zool. Soc. London*, **1**: 1–19.

Irving, L., 1924. Ciliary currents in starfish. *J. Exp. Zool.*, **41**: 115–124.

Millott, N., 1966. A possible function for the axial organ of echinoids. *Nature*, **209**: 594–596.

Unger, H., 1962. Experimentelle und histologische Untersuchungen über Wirkfaktoren aus Nervensystem von *Asterias* (*Marthasterias*) *glacialis* (Asteroidea; Echinodermata). *Zool. Jahrb. Agy. Allg. Zool. Physiol. Tiere*, **69**: 481–536.

Reference: *Biol. Bull.*, **138**: 26–34. (February, 1970)

DUAL CONTROL OF THE LEUCOPHORES IN THE PRAWN, *PALAEMONETES VULGARIS,* BY PIGMENT-DISPERSING AND PIGMENT-CONCENTRATING SUBSTANCES[1]

MILTON FINGERMAN

Department of Biology, Tulane University, New Orleans, Louisiana 70118, and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Examination of the literature dealing with the leucophores of the prawn, *Palaemonetes vulgaris,* reveals that Perkins and Snook (1932) reported that the eyestalks of this organism contain a substance which causes dispersion of its white chromatophoric pigment whereas later Brown (1935) and Hanström (1937) using the same species came to the opposite conclusion, namely that the eyestalk contains a white pigment-concentrating substance. Both Brown and Hanström suggested that dispersion of the white pigment was due to absence of the pigment-concentrating substance. More recently, however, Fingerman and Rao (1969) showed that the eyestalks of *Palaemonetes vulgaris* contain a substance that disperses the white chromatophoric pigment of the fiddler crab, *Uca pugilator,* and another that concentrates this pigment in the crab. But whether these substances would have corresponding actions on the leucophores of *Palaemonetes vulgaris* itself was still a matter of conjecture. Therefore, in view of the results of Fingerman and Rao (1969) and the seemingly conflicting reports of the earlier investigators, it seemed worthwhile to attempt a re-investigation of the endocrine control of the leucophores in this prawn itself.

Materials and Methods

The specimens of *Palaemonetes vulgaris* were collected in the vicinity of Woods Hole, Massachusetts by members of the Supply Department at the Marine Biological Laboratory. I wish to express my appreciation for their efforts. In the laboratory the prawns were maintained in aquaria equipped with running sea water.

Earlier efforts in this laboratory to study the substances controlling migration of the white pigment in this prawn had not been fruitful because of difficulties in designing a satisfactory assay system. In order to accomplish the aim of this investigation it was imperative to devise an assay system that would provide reproducible data. The Hogben and Slome (1931) system of staging chromatophores was employed for quantifying the responses of the leucophores in the epidermis adhering to the portion of the carapace dorsal to the heart. These were the cells whose responses would be investigated. According to the Hogben and Slome scheme, stage 1 represents maximal concentration of the pigment, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions. In his study Brown (1935) had found that the mean stage of the leucophores in eyestalkless prawns was not a uniform value, but instead covered the entire range from 1 to 5. In the search for an adequate assay system to use in the present experiments it was seen that of 165 eyestalkless prawns kept in white containers under an incident illumination of 754 meter-candles for one hour, 22 prawns had a mean leucophore stage of 1, 33 stage 2, 69 stage 3, 27 stage 4, and 14 stage 5, An attempt was made to utilize eyestalkless prawns with their leucophores in stage 3 for the assays because by using such prawns the presence of both pigment-dispersing and -concentrating substances might be detected. Preliminary experiments soon revealed that such an assay system would indeed be suitable. Different groups of eyestalkless prawns selected on the basis of having a mean leucophore stage of 3 responded to the same extract in a very similar manner. Consequently, more detailed experiments were designed and performed with eyestalkless prawns in white containers under an incident illumination of 754 meter-candles and whose leucophores had a mean of stage 3 as the assay animals. A constant intensity of illumination was important. Brown, Sandeen, and Webb (1948) have previously found that the white pigment of *Palaemonetes* shows an increased degree of dispersion with increased illumination.

---

[1] This investigation was supported by Grant GB-7595X from the National Science Foundation.

Extracts to be injected directly into the prawns were prepared in sea water diluted to the osmotic concentration of the prawn's blood (Fingerman and Connell, 1968) which is equivalent to 61.3% of sea water having an original salinity of 35‰. In those experiments where extracts were chromatographed on the gel Sephadex LH-20 prior to assay either 50 eyestalks or 50 supraesophageal ganglia with the circumesophageal connectives attached were first extracted in 0.8 ml ethanol, centrifuged for three minutes at 1815 × g and at 24° C, and then the supernate was applied to the top of the column. The column of Sephadex LH-20 was equilibrated with ethanol which was used as the solvent. The size of the column was 1.5 × 28.0 cm and the void volume was 21 ml. Two milliliter fractions were collected. The flow rate was 30 ml per hour. The alcohol in each fraction was allowed to evaporate and the residue was dissolved in 0.2 ml of the diluted sea water. The injected dose was 0.02 ml.

When extracts of fresh tissues were prepared they were assayed on three or five prawns, as will be described below, but in the chromatography experiments each sample was always assayed on three prawns. For the preparation of extracts directly in isosmotic sea water the desired number of organs was triturated in sufficient saline to provide the desired concentration and then centrifuged under the same conditions as were the ethanol extracts.

Responses to the material obtained after chromatography of the several extracts were expressed in units of the Standard Integrated Response (SIR) as defined by Fingerman, Rao, and Bartell (1967). Calculation of the Standard Integrated Response takes into account both the amplitude and duration of the response.

EXPERIMENTS AND RESULTS

Responses to eyestalk extracts

The object of the first set of experiments was to determine the responses of eyestalkless prawns whose leucophores had a mean Hogben and Slome stage

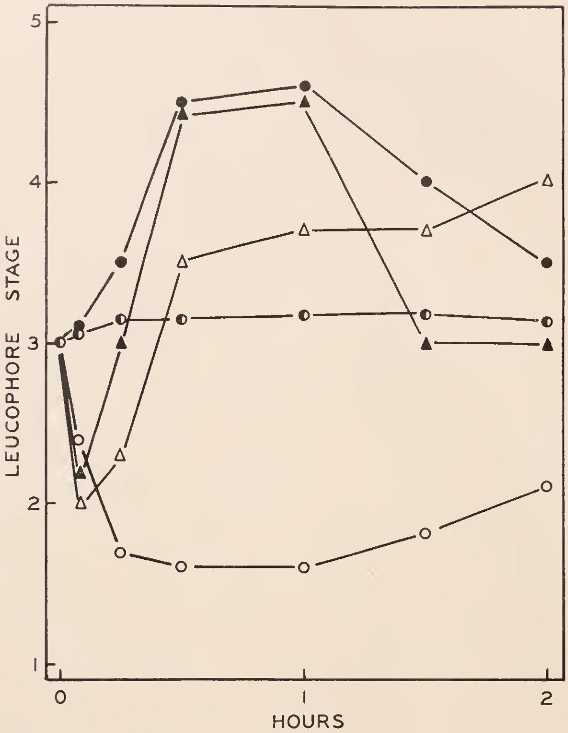


Figure 1. Relationships between the mean leucophore stage of eyestalkless prawns injected with extracts prepared from 8 or 16 eyestalks versus time following injection. Half-filled circles, control; other symbols, mean responses of groups of prawns that received an injection of eyestalk extract. A different extract was injected into each group of prawns. Final extract concentrations is: one-half eyestalk equivalent per dose.

of 3 to extracts of eyestalks in an attempt to resolve the apparently incompatible reports of the effect of these extracts. Eyestalks from 4–8 prawns were triturated in sufficient saline to yield an extract containing one-half of an eyestalk equivalent per dose of 0.02 ml. When four such extracts were assayed, each on five eyestalkless prawns, the effects (Fig. 1) ranged from pigment concentration alone to pigment dispersion alone with the intermediate condition of transitory concentration of the white pigment followed by a pigment-dispersing response. The controls received isosmotic sea water alone.

A possible interpretation of the transitory concentration of the white pigment which was followed by dispersion was that because the extracts were prepared from 8–16 eyestalks in sufficient saline to provide a final concentration of one-half eyestalk equivalent per dose, some eyestalks if extracted singly would evoke only a white pigment-dispersing response while others only a white pigment-concentrating response, and that the mixed response was the result of mixing the two types of eyestalks. To determine whether this interpretation might be the correct one 13 extracts were prepared, each from one eyestalk from 13 different prawns. Each eyestalk was triturated in 0.1 ml isosmotic sea water, to provide a final extract concentration of one-fifth of an eyestalk equivalent per dose. Every one of the 13 extracts was assayed on three eyestalkless prawns. The reason for assaying these extracts on three prawns whereas the previous extracts were assayed on five was that individual eyestalks extracted in only 0.1 ml of fluid did not provide sufficient material for assays on a larger number of prawns because of small losses that occur when extracts are prepared and injected such as, for example, the small volume that always remains in the syringe and needle. The data for three of these 13 extracts are shown in Figure 2. The similarity of the data in Figures 1 and 2 justifies the use of the smaller number of prawns. The results were essentially the same as found with the extracts prepared from more than one eyestalk, some extracts produced dispersion alone, some concentration alone, and some a mixed response. With extracts of single eyestalks the largest responses showing pigment dispersion or concentration alone (Fig. 2) were less than those seen with the extracts prepared from several eyestalks (Fig. 1), reflecting the difference in extract concentration, one-fifth *versus* one-half of an eyestalk equivalent. The results for the entire 13 extracts showed that two produced dispersion alone, four concentration alone, and seven a mixed response. The original hypothesis that the mixed response was due to combining eyestalks that produced either

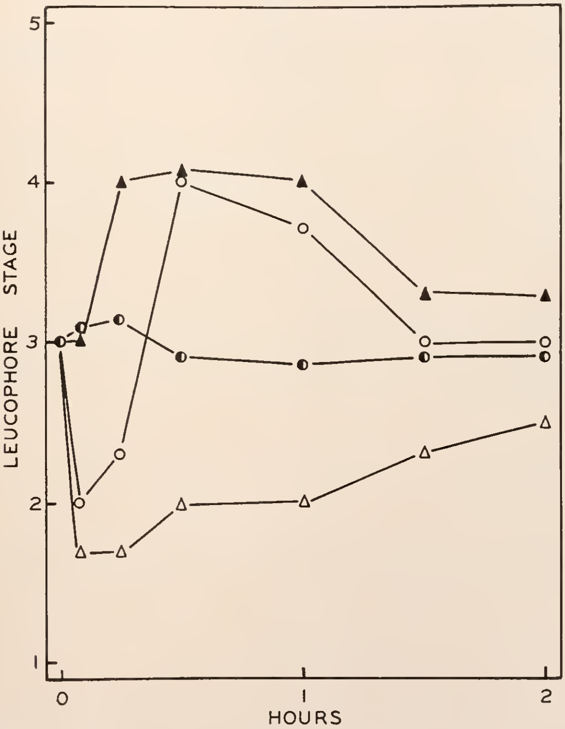


FIGURE 2. Relationships between the mean leucophore stage of eyestalkless prawns injected with extracts prepared from a single eyestalk *versus* time following injection. Symbols same as in Figure 1. Final extract concentration is: one-fifth eyestalk equivalent per dose.

pigment dispersion or concentration alone was not supported by the data with extracts prepared from individual eyestalks.

An experiment utilizing gel filtration on Sephadex LH-20 was then attempted to see if the dispersing and concentrating substances for the white pigment of *Palaemonetes* could be separated from each other by this technique. The averaged results of the experiment, performed three times, are shown in Figure 3. The white pigment-dispersing substance came off the column ahead of the white pigment-concentrating substance, the former peaking in fraction 15 and the latter in fraction 20.

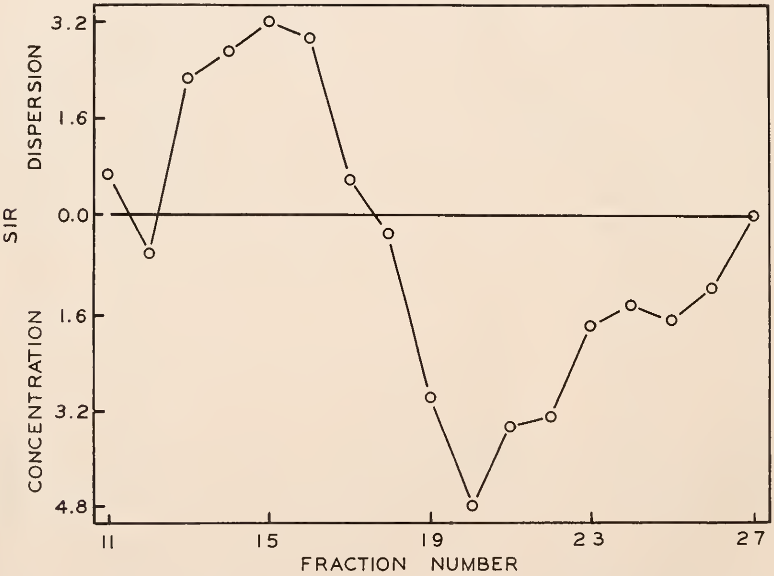


Figure 3. The white pigment-dispersing and -concentrating Standard Integrated Responses (SIR) evoked by the fractions obtained by passing the ethanol-soluble material of the eyestalk through the column of Sephadex LH-20.

Responses to extracts of the supraesophageal ganglia with the circumesophageal connectives attached

The object of this group of experiments was to compare the responses to extracts of the supraesophageal ganglia with the circumesophageal connectives attached with the results presented above for the eyestalks. The first experiment consisted of a determination of the responses of extracts of freshly dissected supraesophageal ganglia plus the circumesophageal connectives in isosmotic sea water. Extracts were prepared from groups of organ complements in a final concentration of one-half organ equivalent (assayed on five prawns) and from single organ complements having a final concentration of one-fifth of an organ equivalent (assayed on three prawns). The experiment was performed four times, always with the same result. Each extract produced only dispersion of the white pigment. The averaged results of the four experiments are presented in Figure 4. The effect of the lower final concentration (one-fifth of an equivalent) of material in the extracts from single organ complements is reflected in the lower response to these extracts compared to the response to the extracts (one-half equivalent) prepared from four organ complements.

One possible interpretation of the results in Figure 4 is that the supraesophageal ganglia and circumesophageal connectives contain a white pigment-dispersing substance to the exclusion of white pigment-concentrating material. An alternative explanation is that a white pigment-concentrating substance is present in these organs, but is completely masked by the white pigment-dispersing substance. The technique of gel filtration on Sephadex LH-20 was employed in an effort to distinguish between these possibilities. A white pigment-concentrating substance was indeed found in addition to the pigment-dispersing one. The experiment was performed three times. The averaged SIR's for the fractions assayed in the three experiments are shown in Figure 5. The peak for the white pigment-concentrating substance in the latter figure occurred in the same fraction as did that in Figure 3 for the eyestalks, fraction 20. The peak of white pigment-dispersing activity occurred in fraction 16 with the supraesophageal ganglia and circumesophageal connectives but in fraction 15 with the eyestalks. However, because the peaks of white pigment-dispersing activity are fairly broad in Figures 3 and 5 there is no significant reason for assuming that the substances are not the same.

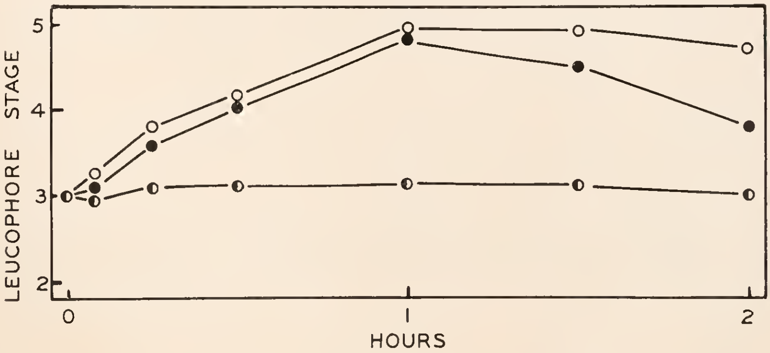


FIGURE 4. Relationships between the mean leucophore stage of eyestalkless prawns injected with extracts of the supraesophageal ganglia with the circumesophageal connectives attached *versus* time following injection. Half-filled circles, control; dots, extracts prepared from single organ complement; circles, extracts prepared from four organ complements. Final extract concentrations are: dots, one-fifth organ equivalent; circles, one-half organ equivalent.

DISCUSSION

The results presented in Figures 1 and 2 showing that some eyestalk extracts produced only dispersion of the white pigment while others produced only concentration provide an explanation for the apparently inconsonant reports of Perkins and Snook (1932) who, as stated above, reported on the one hand that eyestalk extracts caused dispersion of the white pigment of *Palaemonetes* and of Brown

(1935) and Hanström (1937) on the other who reported that these extracts caused concentration. It is conceivable that by chance alone these investigators obtained eyestalks which showed only one effect. The results presented above show clearly that this prawn produces for its own white chromatophoric pigment substances having pigment-dispersing and pigment-concentrating activities. Presumably, all the eyestalks contain both substances but in differing amounts. These substances are certainly antagonistic to each other. The results from the experiment of gel filtration of the supraesophageal ganglia with the circumesophageal connectives attached (Fig. 5) revealed that this antagonism can be strong enough to inhibit completely the response to one of the substances.

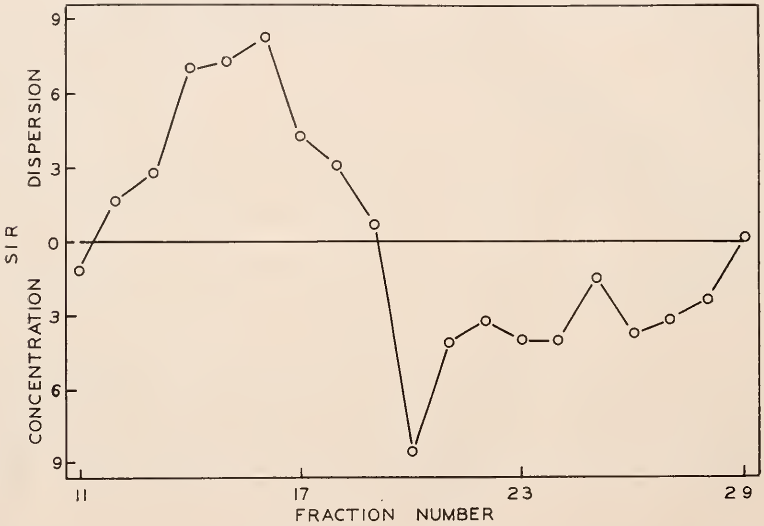


Figure 5. The white pigment-dispersing and -concentrating Standard Integrated Responses (SIR) of eyestalkless prawns evoked by the fractions obtained by passing the ethanol-soluble material of the supraesophageal ganglia with the circumesophageal connectives attached through the column of Sephadex LH-20.

Fingerman and Rao (1969), as stated above, found that the eyestalks of this prawn contain substances that disperse and concentrate the white pigment of the fiddler crab, *Uca pugilator*. These investigators chromatographed the ethanol-soluble material from eyestalks of *Palaemonetes* on a column of Sephadex LH-20 having the same dimensions and under the same conditions as employed in the present investigation, and also collected 2 ml fractions as above. The substance that dispersed the white pigment of the fiddler crab peaked in fraction 15 whereas the substance that concentrated the white pigment of this crab peaked in fraction 20, the same two fractions of the eyestalks that had the maximal corresponding effects on the white pigment of *Palaemonetes* (Fig. 3). It would seem most simply then that the corresponding effects produced by the fractionated extracts of eyestalks from *Palaemonetes* in the prawn and crab were due to the same substances. Fingerman and Rao (1969) had, however, found that although dispersion of the white pigment in the fiddler crab could have been due to the same substance being present in the ethanol-soluble material from the eyestalks of both the crab and the prawn, the substances in the two extracts that concentrated the crab's white pigment were different from each other. The substance in the ethanol-soluble material from the crab's eyestalks that concentrated its white pigment was a larger molecule than the substance from the eyestalk of the prawn that concentrated the white pigment of the fiddler crab, although not as large as the white pigment-dispersing substance.

The conclusion of Brown (1935) and Hanström (1937) that dispersion of the white chromatophoric pigment in *Palaemonetes* was due not to a white pigment-dispersing substance, but merely to the absence of white pigment-concentrating hormone is not supported by the data presented above which revealed that the prawn possesses both substances. From a comparative viewpoint it is interesting that whereas the white pigment-dispersing and -concentrating responses evoked by the fractionated extracts of eyestalks from *Palaemonetes* in *Palaemonetes* (Fig. 3) and *Uca* (Fingerman and Rao, 1969) would appear to have been due to the same substances, Fingerman and Couch (1967) in comparing the responses of the erythrophores of these two crustaceans to aqueous extracts of freshly dissected organs from *Palaemonetes* concluded that the substances that dispersed and concentrated the red pigment in *Palaemonetes* were different from those in *Palaemonetes* that had corresponding effects in *Uca*.

SUMMARY

1. The prawn, *Palaemonetes vulgaris*, produces substances that cause dispersion and concentration of its white chromatophoric pigment.

2. Injection of extracts prepared from one or more eyestalks triturated directly in isosmotic sea water produced concentration of the white pigment alone, dispersion alone, or concentration followed by dispersion. In contrast, similarly prepared fresh extracts of the supraesophageal ganglia with the circumesophageal connectives attached always produced white pigment dispersion alone.

3. Chromatography of ethanol extracts of eyestalks and as well as supraesophageal ganglia with the circumesophageal connectives attached on the gel Sephadex LH-20 always yielded both the white pigment-dispersing substance and the -concentrating substance. The former preceded the latter off the gel column.

4. The responses obtained with extracts prepared directly in isosmotic sea water were interpreted as having been due to the relative quantities of the white pigment-dispersing and -concentrating substances present in the organs in addition to an antagonism between these chromatophorotropins.

LITERATURE CITED

Brown, F. A., Jr., 1935. Control of pigment migration within the chromatophores of *Palaemonetes vulgaris*. *J. Exp. Zool.*, **71**: 1–14.

Brown, F. A., Jr., M. I. Sandeen and H. M. Webb, 1948. The influence of illumination on the chromatophore system of *Palaemonetes vulgaris*. *Anat. Rec.*, **101**: 733.

Fingerman, M., and P. M. Connell, 1968. The role of cations in the actions of the hormones controlling the red chromatophores of the prawn, *Palaemonetes vulgaris*. *Gen. Comp. Endocrinol.*, **10**: 392–398.

FINGERMAN, M., AND E. F. COUCH, 1967. Differentiation of chromatophorotropins from the prawn, *Palaemonetes vulgaris*, and the fiddler crab, *Uca pugilator*. *J. Exp. Zool.*, **165**: 183–194.

FINGERMAN, M., AND K. R. RAO, 1969. A comparative study of leucophore-activating substances from the eyestalks of two crustaceans, *Palaemonetes vulgaris* and *Uca pugilator*. *Biol. Bull.*, **136**: 200–215.

FINGERMAN, M., K. R. RAO AND C. K. BARTELL, 1967. A proposed uniform method of reporting response values for crustacean chromatophorotropins: the Standard Integrated Response. *Experientia*, **23**: 962.

HANSTRÖM, B., 1937. Die Sinusdrüse und der hormonal bedingte Farbwechsel der Crustaceen. *Kgl. Svenska Vetenskapsakad. Handl.* **16** Nr. 3: 1–99.

HOGBEN, L., AND D. SLOME, 1931. The pigmentary effector system. VI. The dual character of endocrine co-ordination in amphibian colour change. *Proc. Roy. Soc. London, Series B.*, **108**: 10–53.

PERKINS, E. B., AND T. SNOOK, 1932. The movement of pigment within the chromatophores of *Palaemonetes*. *J. Exp. Zool.*, **61**: 115–128.

Reference: *Biol. Bull.*, **138**: 35–46. (February, 1970)

COMBINED EFFECTS OF SALINITY AND TEMPERATURE ON THE FEEDING, REPRODUCTIVE, AND SURVIVAL RATES OF *EUPLEURA CAUDATA* (SAY) AND *UROSALPINX CINEREA* (SAY) (PROSOBRANCHIA: MURICIDAE)[1]

JOHN J. MANZI

Bureau of Commercial Fisheries, Biological Laboratory, Milford, Connecticut 06460

The marine prosobranch gastropods, *Eupleura caudata* (Say) and *Urosalpinx cinerea* (Say), are among the most serious predators of the Eastern oyster (*Crassostrea virginica*). The damage inflicted by these drills is estimated to be in the millions of dollars per year (Nelson, 1931; Galtsoff, Prytherch, and Engle, 1937). Both species are widely distributed along the coastal waters of the eastern United States, often inhabiting areas where salinity and temperature vary with seasonal and tidal cycles. The natural exposure of these gastropods to a fluctuating environment has led investigators to study the influence of salinity or temperature on their physiological activities.

Many investigators believed that drills begin to feed only above a certain critical temperature (Federighi, 1931a; Galtsoff *et al.*, 1937; Cole, 1942; Adams, 1947; Hancock, 1954). After the proposal of physiological speciation in oyster drills (Stauber, 1950; Loosanoff and Davis, 1951), however, many authorities accepted the suggestion that no one critical temperature exists at which all oyster drills begin feeding (Hanks, 1957; Franz, 1966). The effect of salinity on feeding rates of oyster drills has been investigated only superficially in field studies (Haskin, 1935; Sizer, 1936).

The initiation and rate of egg capsule deposition appear to be greatly influenced by temperature (Federighi, 1931a; Cole, 1942; Hancock, 1959; Franz, 1966, 1967), although other factors, such as food, availability of suitable substratum, and population density, may be contributory regulators of spawning intensity (Stauber, 1943; Carriker, 1955; Galtsoff *et al.*, 1937). Loosanoff and Davis (1951) reported that a single species-wide critical temperature for egg capsule deposition does not exist for *U. cinerea.* This situation could be expected from populations collected from different geographical areas but it also appears to hold true among populations collected from the same area. The effect of salinity on egg capsule deposition has been less thoroughly investigated, although available reports suggest that salinity is a regulator of spawning activities of drills (Federighi, 1931a; Stauber, 1943; Carriker, 1955).

Oyster drills appear to remain active over a relatively wide temperature range (Sizer, 1936; Hanks, 1957); however, the effect of salinity on drill survival is relatively unknown. Many reports on the effect of salinity on oyster drills are conflicting, particularly concerning the minimum salinity at which the drills can survive (Federighi, 1931b; Sizer, 1936; Griffith and Engle, 1962).

---

[1] Adapted from portions of a thesis submitted to Southern Connecticut State College in partial fulfillment of the requirements for the degree of Master of Science.

Sizer (1936), on the basis of his experimental results, concluded that *U. cinerea* are active only between 20 and 30‰. Galtsoff *et al.* (1937) found drills active in Delaware Bay between 15 and 29‰ at summer temperatures. Stauber (1943) concluded, from field observations and laboratory experiments, that *U. cinerea* remain active over a fairly wide salinity range and that they can move in salinities as low as 8‰ at low winter temperatures.

Many of the conclusions concerning the effects of temperature and salinity on oyster drills are conflicting. Although differences in individual experimental procedures and differences in test populations attributable to physiological speciation may account for some of these disparities, it is now evident that the effects of salinity and temperature cannot be analyzed by monofactorial experiments. Kinne (1964) recognized that monofactorial experiments on the effects of a single environmental variable may yield results that are ecologically invalid. He suggested that a polyfactorial approach be used whenever possible to analyze the influence of environmental stimuli.

Data are scant on the combined effects of two environmental stimuli on oyster drills. Stauber (1943), who was the first to evaluate the influence of temperature on the response of oyster drills to salinity, concluded that the survival of drills exposed to low salinities increased as the temperature was lowered and decreased as the temperature was increased. More recently, the combined effect of temperature and salinity on larval stages was determined for *C. virginica* and *Mercenaria mercenaria* (Davis and Calabrese, 1964) and for *Mulinia lateralis* (Calabrese, 1969). They concluded that the effects of these environmental factors were interrelated; changes of either factor influenced the effect of the other. The present bifactorial investigation was initiated to determine the combined effect of salinity and temperature on the feeding, reproduction, and survival of the two oyster drill species indigenous to Long Island Sound.

Materials and Methods

Collection of animals

The oyster drills, *U. cinerea* and *E. caudata,* were collected in Long Island Sound in the vicinity of the Norwalk Islands, Norwalk, Connecticut. This area is extensively used for farming oysters and is characterized by relatively shallow depths, moderate currents, and a variety of bottom substrata (sand, silt, gravel). It is also one of the few known areas on the Connecticut shore where relatively large numbers of both drill species can be obtained. During July and August 1967, when the majority of the drills were collected, the bottom salinities averaged 27‰ and the temperatures 22.2° C.

Two methods were employed for capturing the drills. The first consisted of setting drill traps in the relatively shallow water (3.6 m) off the southwest corner of Norwalk, town lot 19. The traps of chicken wire made into flat 64 × 30 cm bags were baited with 1-year-old oysters. At 2-week intervals the traps were retrieved, drills collected, and the traps freshly baited and reset.

Although this method provided approximately equal numbers of both drill species, it did not yield the large numbers of drills required. The second and more efficient method consisted of hand-picking drills from bottom material brought to the surface by standard oyster dredges. Norwalk town lots 35, 50, 80, and 207 were dredged by the oyster boat, "Cultivator" (Bloom Bros.), and the Bureau of Commercial Fisheries research vessel, "Shang Wheeler." This procedure proved to be a rapid and reliable means of drill collection.

Oyster spat (3–13 months old) were dredged from the same area. Some of the spat were from natural set (local and Fishers Island, New York) and some from commercial hatchery set (Bloom Bros., Stratford, Connecticut, and Vanderborgh-Radel, Oyster Bay, New York).

Conditioning

The drills were separated by species in the laboratory and placed in separate 60-liter fiberglass aquaria. The aquaria, arranged on a laboratory water table, were each supplied with a separate continuous flow of sea water at normal temperature (22.5° C) and salinity (26.5‰). Clusters of young oyster spat were added to each aquarium to provide food and substrate for the drills during the 1-week acclimatization period. chosen [illegible]

To condition the drills to the various salinity-temperature combinations, the sea water in which the drills were kept was gradually brought to the appropriate temperature and salinity. The temperature of the water entering the conditioning aquarium containing the drills to be exposed to a series of salinities at a particular temperature was either increased or decreased 1° C per day until the desired temperature was reached. The temperature was then held constant and the salinity was decreased by 1‰ per day until the desired salinity was reached.

As each salinity level was attained, groups of drills were removed from the conditioning aquaria and placed into a holding aquarium maintained at that temperature and salinity, until one holding aquarium was established at each of the four salinities to be tested. After 1 week in these holding aquaria and before the experiments the drills were live-sexed by the method described by Hargis (1957) and segregated by sex in separate aquaria. To insure that the drills were sexually mature, only females 20 mm or larger and males with a well-developed penis were used in the experiments.

Groups of 20 drills (14 females and 6 males) were then removed for use in the experimental trays. The remainder were kept in the holding aquarium throughout the experiment to supply conditioned drills to replace those that died during the experiment. All aquaria were drained and cleaned at 1-week intervals.

The young *C. virginica* used as prey were not conditioned; they were held in aquaria supplied with running water at normal temperature and salinity.

Experimental procedure

Each laboratory at the Milford Biological Laboratory of the Bureau of Commercial Fisheries is supplied with running hot and cold sea water, as well as cold fresh well water. By combining streams of water from these three sources a variety of temperatures and salinities can be obtained. An apparatus similar to the one described by Loosanoff (1949) and Loosanoff and Smith (1950) was used to maintain a flow of water at the desired temperature and salinity. The required combinations of temperature and salinity were obtained by adjusting the rate of flow from each of the three water sources to several polyethylene mixing cylinders placed above a laboratory water table. The flow of water to the cylinders was adjusted so that the surplus continuously drained through the overflows, thus keeping the water level and head pressure constant in all cylinders. A Y-tube at the outlet from each cylinder provided two separate streams of water which supplied two fiberglass trays (35 cm × 48 cm × 11.5 cm), each containing 20 oyster drills. The rate of flow to each tray was approximately 2 liters per minute. The trays were covered with small-mesh nylon netting to prevent the drills from escaping.

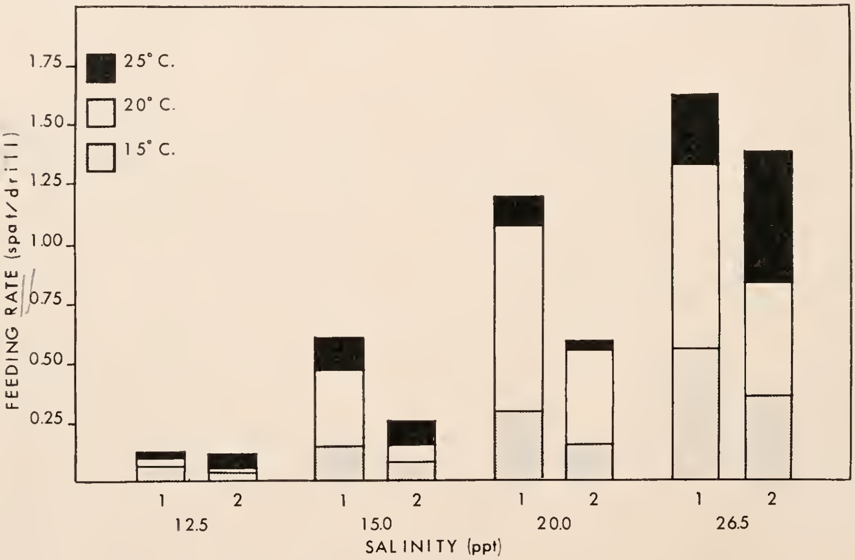


Figure 1. Feeding rates of *Urosalpinx cinerea* (1) and *Eupleura caudata* (2), per 10-day trial, on young *Crassostrea virginica*, at a series of controlled temperatures and salinities.

Three experiments were conducted to determine the combined effects of salinity and temperature on certain fundamental activities of *U. cinerea* and *E. caudata*. The same salinities (12.5, 15, 20, and 26.5 ± 1‰) were repeated in each experiment, but the temperature was different. Salinity was measured daily by hydrometers calibrated at 17.5° C and corrected for temperature with the hydrographical tables of Knudsen (1901). Each experiment consisted of five 10-day trials; thus, the total elapsed time was 150 days. The salinity tolerance of the drills was studied at temperatures of 15, 20, and 25 ± 1° C.

The four salinities used in each of the three experiments were established by the method previously described. Two trays were kept at each salinity; one contained 20 *U. cinerea* and the other, 20 *E. caudata*. Both trays were stocked with approximately 60 spat (*C. virginica*) on 5 to 6 clusters. The trays were examined daily and were drained, cleaned, and restocked at 10-day intervals. At the end of each 10-day trial the rates of feeding, ovipositing, and survival were determined. Consumed prey were distinguished from other prey mortalities by a simple criterion—the presence of a perforation through one of the valves. Dead drills were easily identified by the putrifying tissue usually observable in the shell aperture. Unusual podial extension or retraction, however, often indicated morbidity, and drills were tested for viability by pin-prick or exposure to water of normal temperature. Reproductive rates were measured by the numbers of egg capsules deposited during each 10-day trial. Counts of egg numbers in each capsule were also made. With this procedure both drill species were studied simultaneously in the presence of the same prey species at the salinities and temperatures established for each experiment.

RESULTS AND DISCUSSION

Effects of salinity and temperature on feeding

Feeding rates at the 12 salinity-temperature combinations were recorded as the mean number of oyster spat consumed per drill per 10-day trial (Table I). At 15° C the feeding rates of both drill species increased from minimal (0.05 and 0.01) at a salinity of 12.5‰ to moderate (0.54 and 0.29) at 26.5‰. At the lower salinities (12.5 and 15‰) the drills remained relatively inactive, exhibiting little tendency to move toward or attack prey; however, they were firmly attached to the trays or shell clusters. As the salinity increased, general drill activity increased.

Feeding rates at 20° C were significantly higher at all salinities than at 15° C (Table I). At 12.5‰ the number of prey consumed per drill per trial was still

TABLE I

Feeding rates of Urosalpinx cinerea *and* Eupleura caudata *during five 10-day trials at a series of controlled water temperatures and salinities**

Drill species and salinity ‰	Average no. of prey consumed per trial			Average no. of prey consumed per drill per trial		
	Temperature °C			Temperature °C		
	15.0	20.0	25.0	15.0	20.0	25.0
U. cinerea						
12.5	1.0	1.8	2.0	0.05	0.09	0.10
15.0	2.8	9.4	10.6	0.14	0.47	0.53
20.0	5.4	21.0	23.2	0.27	1.05	1.16
26.5	10.8	26.0	33.2	0.54	1.30	1.66
E. caudata						
12.5	0.2	0.6	1.6	0.01	0.03	0.08
15.0	1.4	2.8	4.4	0.07	0.14	0.22
20.0	2.8	10.8	11.4	0.14	0.54	0.57
26.5	5.8	16.2	27.0	0.29	0.81	1.35

* In each trial 20 drills were held in a tray with approximately 60 *Crassostrea virginica* spat.

low (0.09 and 0.03) but the combined increase in feeding of both drill species with each increase in salinity was higher than it was at 15° C. At normal salinity (26.5‰) the feeding rates of both drills were relatively high (1.30 and 0.81).

At all salinities the drills held at 25° C showed the highest feeding rates (Table I). The rate of prey consumption increased from 0.10 and 0.08 oyster spat per drill per trial at 12.5‰ to the maximum observed rates of 1.66 and 1.35 at normal salinity (26.5‰). At no time during the experiment did the drills consume all the prey available; thus, availability of food did not limit feeding.

At each salinity the feeding rates of both species increased with each increase in temperature; also, at each temperature they increased with each increase in salinity. The increased feeding rates at high temperatures were expected (Hanks, 1957), but the increased feeding rates with each increase in salinity did not agree with the findings of Haskin (1935). He found that the rate of feeding was not appreciably altered within the salinity range of his study (10 to 25‰). Results of the present series of experiments do support Haskin's conclusion that feeding stops below 10–12‰, but they also indicate that the feeding rates of both species of drills are appreciably altered by salinity at all three temperatures studied. The feeding rates of both *U. cinerea* and *E. caudata* at normal salinity (26.5‰) at each temperature agreed with previous feeding rates established for *U. cinerea* (Hanks, 1957) and *E. caudata* (Manzi, in preparation).

A comparison of the feeding rates of the two drills showed that *U. cinerea* was consistently the more voracious oyster predator at all temperature-salinity combinations studied (Fig. 1). At the lower combinations *E. caudata* consumed young oyster spat at approximately one-third of the rate exhibited by *U. cinerea.* At the higher combinations, however, the disparity was considerably reduced. These observations indicate that *U. cinerea* is probably the more serious predator of *C. virginica;* it should not be inferred, however, that *U. cinerea* is the more voracious predator on other common prey of the two drills.

Both drill species were cannibalistic in many of the experimental trays. The rate of cannibalism increased as the feeding rates increased, and the highest incidence of cannibalism was at the optimum feeding conditions (25° C, 26.5‰). Although cannibalism has been observed in both drill species previously (Flower, 1954; C. L. MacKenzie, Jr., Bureau of Commercial Fisheries, Milford, Connecticut, personal communication), findings of the present study confirm my previous observations (Manzi, in preparation) of cannibalism throughout the limits of the drill's feeding range in the presence of alternative food sources. Both drill species were cannibalistic, but *E. caudata* was the more so, which may have had a significant bearing on the results observed. In all cases where active cannibalism was observed the predators were always female drills.

Statistical evaluation of the results by "t" tests revealed that the differences in feeding rates at the progressively increasing salinities were significant (95% confidence level, $t > 4.604$ with $d.f. = 4$), for each species of drill, at all temperature levels. At 12.5‰ salinity "t" tests showed no significant difference in feeding rates at temperatures of 15, 20, and 25° C. At all higher salinities, differences in feeding rates at the three different temperatures were significant at the 95% confidence level.

Effect of salinity and temperature on reproduction

Neither species of oyster drill deposited egg capsules at salinities of 15‰ or lower (Table II). At 20‰ *U. cinerea* did not deposit egg capsules at 15° C although *E. caudata* deposited a few (13). At higher temperatures both species of drills deposited egg capsules, and both species deposited more capsules at 25° C than at 20° C (Table II). At a salinity of 26.5‰ both drills deposited egg capsules at all temperatures tested (15, 20, and 25° C).

Within the range of temperature and salinity at which oviposition occurs, the number of capsules deposited by *E. caudata* and *U. cinerea* increased with each increase in temperature and salinity (Fig. 2). These results indicate that the effects of temperature and salinity on reproduction of oyster drills are interrelated, and confirm the conclusions of Haskin (1935) and Stauber (1943) that oyster drills can survive in areas where they cannot reproduce.

TABLE II

Reproductive rates of Urosalpinx cinerea *and* Eupleura caudata *during five 10-day trials at a series of controlled water temperatures and salinities**

Drill species and salinity ‰	Total no. of egg capsules deposited			Average no. of eggs per capsule		
	Temperature °C			Temperature °C		
	15.0	20.0	25.0	15.0	20.0	25.0
U. cinerea						
12.5	0	0	0	0	0	0
15.0	0	0	0	0	0	0
20.0	0	135	174	0	11.6	13.2
26.5	108	261	271	9.8	10.8	10.0
E. caudata						
12.5	0	0	0	0	0	0
15.0	0	0	0	0	0	0
20.0	13	21	108	18.6	17.8	20.4
26.5	33	158	312	19.4	18.8	19.6

* 20 drills (14 females and 6 males) were used in each trial.

The reproductive rates of *U. cinerea* and *E. caudata* were not greatly different; *U. cinerea,* however, deposited slightly more egg capsules than *E. caudata* at most of the temperature-salinity combinations within the range at which oviposition occurred.

The number of eggs per capsule did not vary with temperature and salinity. The number of eggs per capsule differed significantly, however, between species. *U. cinerea* deposited an average of 11.1 eggs per capsule, with a range of means from 9.8 to 13.2 eggs per capsule (Table II), and *E. caudata* deposited almost twice as many, an average of 19.1 and a range from 17.8 to 20.4 eggs per capsule. The lack of variation in the number of ova per capsule for each drill species at the different temperature-salinity combinations could be due to an effect on production of either ripe ova or egg capsules. The increased reproductive rate

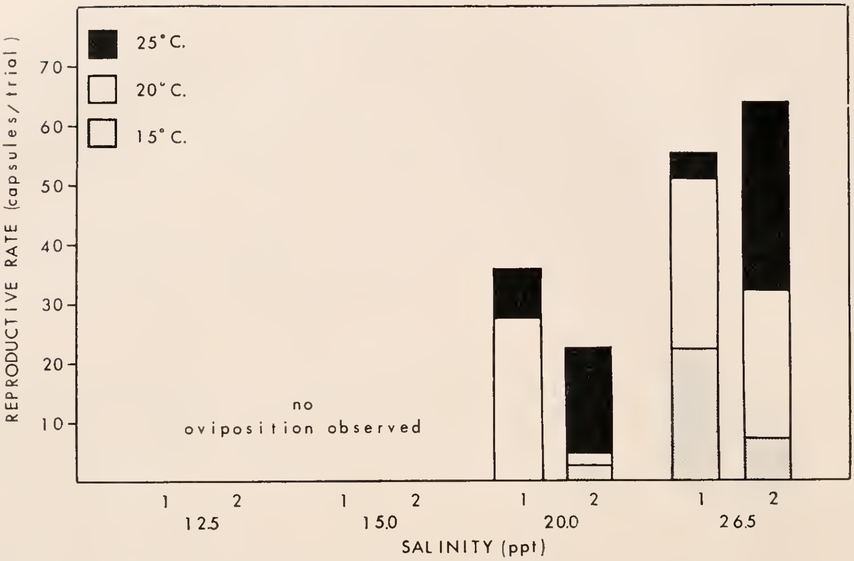


FIGURE 2. Reproductive rates of *Urosalpinx cinerea* (1) and *Eupleura caudata* (2), per 10-day trial, at a series of controlled temperatures and salinities.

at higher temperatures and salinities, however, may be the result of a generally increased metabolic rate affecting both ripening of ova and production of egg capsules.

Effects of salinity and temperature on survival

Drill mortalities were recorded as the mean number of drills dying during a 10-day trial, as well as the percentage mortality per trial (Table III). The lowest

TABLE III

Mortality rates of Urosalpinx cinerea *and* Eupleura caudata *during five 10-day trials at a series of controlled water temperatures and salinities*

Drill species and salinity ‰	Average no. of drill deaths per trial			Per cent drill mortality per trial		
	Temperature °C			Temperature °C		
	15.0	20.0	25.0	15.0	20.0	25.0
U. cinerea						
12.5	1.6	3.8	6.8	8	19	34
15.0	2.0	2.4	3.4	10	12	17
20.0	0.8	1.0	1.4	4	5	7
26.5	0.2	0.4	1.2	1	2	6
E. caudata						
12.5	2.2	4.0	8.2	11	20	41
15.0	3.0	3.6	4.4	15	18	22
20.0	0.8	1.0	2.4	4	5	12
26.5	0.4	0.6	1.4	2	3	7

death rate at each salinity was at 15° C. The highest mortalities of *U. cinerea* and *E. caudata* at this temperature occurred at 15‰ salinity (10 and 15%, respectively). At normal salinity and 15° C mortalities of the two species were lower (1 and 2%, respectively) than for any other temperature-salinity combination tested.

At 20° C and 12.5‰ salinity *U. cinerea* displayed 19% mortality and *E. caudata* 20%. Mortality at this temperature decreased with each increase in salinity and the lowest mortality at this temperature was at 26.5‰; *U. cinerea,* 2% and *E. caudata,* 3%.

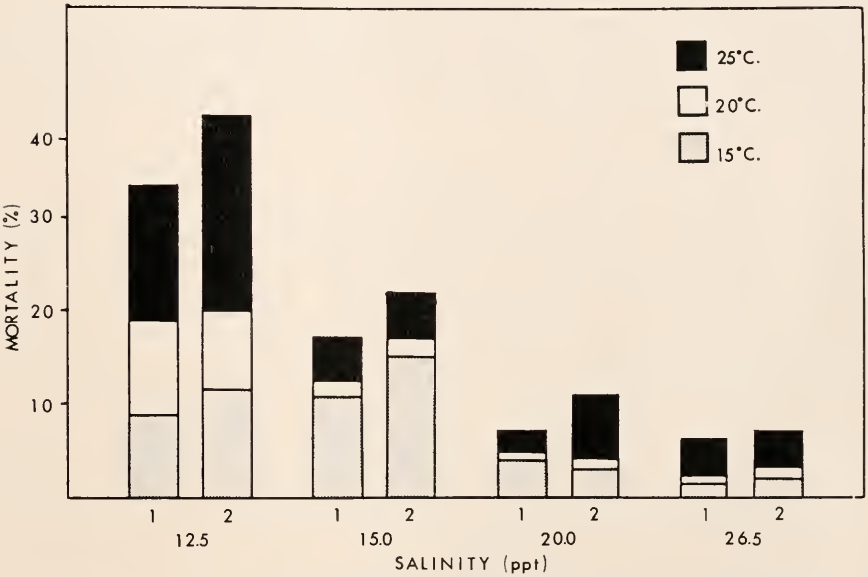


FIGURE 3. Mortalities of *Urosalpinx cinerea* (1) and *Eupleura caudata* (2) at a series of controlled temperatures and salinities.

The highest mortality at all salinities was at 25° C. At this temperature and 12.5‰ salinity, *U. cinerea* and *E. caudata* exhibited mortalities of 34 and 41%, respectively—higher than at any other temperature-salinity combination tested. Again, the mortalities decreased as the salinity increased, and the lowest mortality at this temperature was at 26.5‰.

The ability of oyster drills to survive, particularly at low salinities, was markedly affected by temperature. Both species of drills exhibited progressively higher mortalities as the temperature was increased at each salinity, and at 12.5‰ the mortality rates were approximately four times higher at 25° C than at 15° C (Fig. 3).

These observations may explain the survival of drills in areas where seasonal fluctuations in salinity are wide. In late winter and early spring, when the salinity of coastal waters is lowest, the water temperature is still low enough for drills to withstand the low salinities for some time. *E. caudata,* however, was less tolerant than *U. cinerea* to lower salinities at all temperatures studied; this feature could explain the absence of *E. caudata* in intertidal zones and other areas with wide fluctuations in salinity where *U. cinerea* is frequently found.

At the temperature-salinity combinations where feeding rates were moderate to high, cannibalism accounted for a relatively large part of the drill mortalities. At the high temperature-low salinity combinations, where the mortalities were highest, cannibalism was rare and did not significantly affect the mortalities recorded.

I thank Dr. James E. Hanks, Dr. Charles Steinmetz, Jr., and Mr. Chester Bosworth for their continued assistance and encouragement during the period of my graduate studies and the preparation of this paper, and Mr. Harry C. Davis, Dr. Robert A. Murchelano, and Dr. Anthony Calabrese for their counsel and constructive criticism of the typescript.

SUMMARY

1. At all temperatures studied the limited feeding of *Urosalpinx cinerea* and *Eupleura caudata* at 12.5‰ indicates that this salinity is near the lower limit for feeding.

2. The feeding rates increased with each increase in temperature and salinity.

3. The maximum feeding rates were at the highest temperature-salinity combination studied (25° C, 26.5‰).

4. At all temperature and salinity combinations *U. cinerea* consumed more oyster spat than did *E. caudata*. Given equal populations, therefore, *U. cinerea* is the more important of the two species as a predator of *Crassostrea virginica*.

5. Both species of drills exhibited cannibalism in the presence of alternative food sources, but *E. caudata* did so to a greater extent than *U. cinerea*.

6. Cannibalism increased as the feeding rate increased, and the highest incidence of cannibalism was at optimum feeding conditions.

7. In all instances of cannibalism the predators were female drills.

8. The mortality rates of both species of drills increased with increasing temperature and decreasing salinity.

9. Mortality was highest at the highest temperature and lowest salinity combination (25° C, 12.5‰) and lowest at the lowest temperature and highest salinity (15° C, 26.5‰).

10. *E. caudata* was less tolerant than *U. cinerea* to low salinities at all temperatures.

11. *E. caudata* began ovipositing at 15° C and 20‰, and *U. cinerea* at 20° C and 20‰.

12. The number of egg capsules deposited by each species of drill increased with each increase in temperature and salinity; the maximum number was deposited at 26.5‰ at all temperatures studied.

13. The number of eggs in each capsule did not appear to be affected by temperature or salinity.

LITERATURE CITED

ADAMS, J. R., 1947. The oyster drill in Canada. *Fish. Res. Board Can., Progr. Rept.,* **37**: 14–18.

CALABRESE, A., 1969. Individual and combined effects of salinity and temperature on embryos and larvae of the coot clam, *Mulinia lateralis* (Say). *Biol. Bull.,* **137**: 417–428.

CARRIKER, M. R., 1955. Critical review of the biology and control of oyster drills *Urosalpinx* and *Eupleura. U. S. Fish Wildlife Serv., Spec. Sci. Rept.,* **148**: 1–150.

COLE, H. A., 1942. The American whelk tingle, *Urosalpinx cinerea* (Say), on British oyster beds. *J. Mar. Biol. Ass. U. K.,* **25**: 477–508.

DAVIS, H. C., AND A. CALABRESE, 1964. Combined effects of temperature and salinity on development of eggs and growth of larvae of *M. mercenaria* and *C. virginica. Fish. Bull., U. S. Fish Wildlife Serv.,* **63**: 643–655.

FEDERIGHI, H., 1931a. Studies on the oyster drill (*Urosalpinx cinerea* Say.) *Bulletin U. S. Bureau of Fisheries,* **47**: 83–115.

FEDERIGHI, H., 1931b. Salinity death-points of the oyster drill snail, *Urosalpinx cinerea* Say. *Ecology,* **12**: 346–353.

FLOWER, F. B., 1954. A new enemy of the oyster drill. *Science,* **120**: 231–232.

FRANZ, D. R., 1966. Some studies on physiological variation among populations of the oyster drill, *Urosalpinx cinerea. Proc. Nat. Shellfish. Ass.,* **56**: 3.

FRANZ, D. R., 1967. Further studies on physiological variation among populations of oyster drills. *Proc. Nat. Shellfish. Ass.,* **57**: 2.

GALTSOFF, P. S., H. F. PRYTHERCH AND J. B. ENGLE, 1937. Natural history and methods of controlling the common oyster drills (*Urosalpinx cinerea* Say and *Eupleura caudata* (Say). *U. S. Bureau of Fisheries Circ.,* No. **25**: 1–24.

GRIFFITH, G. W., AND J. B. ENGLE, 1962. Role of salinity in the distribution of the oyster drill, *Urosalpinx cinerea,* in the Rappahannock River, Virginia. *Unpubl. Rept. U. S. Fish Wildlife Serv.,* Oxford, Maryland. (Available from Bureau of Commercial Fisheries Library, Milford, Connecticut).

HANCOCK, D. A., 1954. The destruction of oyster spat by *Urosalpinx cinerea* (Say) on Essex oyster beds. *J. Cons. Perm. Int. Explor. Mer.,* **20**: 186–196.

HANCOCK, D. A., 1959. The biology and control of the American whelk tingle *Urosalpinx cinerea* (Say) on English oyster beds. *Ministry of Agriculture, Fisheries and Food, Fishery Investigations, London, Series II,* **22**: 1–66.

HANKS, J. E., 1957. The rate of feeding of the common oyster drill, *Urosalpinx cinerea* (Say), at controlled water temperatures. *Biol. Bull.,* **112**: 330–335.

HARGIS, W. J., 1957. A rapid live-sexing technique for *Urosalpinx cinerea* and *Eupleura caudata,* with notes on previous methods. *Limnol. Oceanogr.,* **2**: 41–42.

HASKIN, H. H., 1935. Investigations into the boring and reproductive activities of the oyster drills *Urosalpinx cinerea* (Say) and *Eupleura* sp. *Unpubl. Rept. U. S. Bur. Fish.,* Washington, D. C. (Available from Bureau of Commercial Fisheries Library, Milford, Connecticut).

KINNE, O., 1964. The effects of temperature and salinity on marine and brackish water animals. II. Salinity and temperature-salinity combinations. *Oceanogr. Mar. Biol. Annu. Rev.,* **2**: 281–339.

KNUDSEN, M., 1901. *Hydrographical Tables.* Copenhagen, G. E. C. Gad. 1901. [2nd ed. 1931, pp. 1–63, photoprinted by Tutein and Koch.]

LOOSANOFF, V. L., 1949. Method for supplying a laboratory with warm sea water in winter. *Science,* **110**: 192–193.

LOOSANOFF, V. L., AND H. C. DAVIS, 1951. Behavior of drills of different geographical districts at the same temperature. *Unpubl. Rept. U. S. Fish Wildlife Serv.,* Milford. Conn. (Available from Bureau of Commercial Fisheries Library, Milford, Connecticut).

LOOSANOFF, V. L., AND P. B. SMITH, 1950. Apparatus for maintaining several streams of water of different constant salinities. *Ecology,* **31**: 473–474.

Nelson, J. R., 1931. Trapping the oyster drill. *Bulletin New Jersey Agricultural Experimental Station,* **583**: 1–12.

Sizer, I. W., 1936. Observations on the oyster drill with special reference to its movement and to the permeability of its egg case membrane. *Unpubl. Rept. U. S. Bur. Fish.,* Washington, D. C. (Available from Bureau of Commercial Fisheries Library, Milford, Connecticut).

Stauber, L. A., 1943. Ecological studies on the oyster drill, *Urosalpinx cinerea,* in Delaware Bay, with notes on the associated drill, *Eupleura caudata,* and with practical consideration of control methods. *Unpubl. Rept. Oyster Res. Lab.,* N. J. (Available from L. A. Stauber, Rutgers University, New Brunswick, New Jersey).

Stauber, L. A., 1950. The problem of physiological species with special reference to oysters and oyster drills. *Ecology,* **31**: 109–118.

Reference: *Biol. Bull.*, **138**: 47–55. (February, 1970)

INDUCTION OF METAMORPHOSIS BY ECDYSONE ANALOGUES: *DROSOPHILA* IMAGINAL DISCS CULTURED *IN VIVO*

JOHN H. POSTLETHWAIT AND HOWARD A. SCHNEIDERMAN[1]

Marine Biological Laboratories, Woods Hole, Massachusetts 02543, and Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106

The imaginal discs of *Drosophila* provide an increasingly attractive system for the study of development (see *e.g.*, Gehring, 1968). One such developmental problem is the hormonal control of metamorphosis. This problem and others could be investigated more easily if metamorphosis could be induced *in vitro*. The early literature on the somewhat disappointing attempts at *in vitro* culture of insect tissues has been reviewed by Day and Grace (1959). Imaginal discs cultured *in vitro* without ring glands do not metamorphose, whereas, when ring glands are added to the medium, the discs will metamorphose (Kuroda and Yamaguchi, 1956; Gottschewski, 1960; Schneider, 1964, 1966). Experiments with crystalline molting hormone, ecdysone, have been less successful. Oberlander and Fulco (1967) and Oberlander (1969) have reported that the only observable effects of ecdysone, ecdysterone, or inokosterone on wing discs of the moth *Galleria mellonella* cultured *in vitro* were tracheal growth and occasional elongation. Burdette, Hanley and Grosch (1968) cultured eye-antennal discs of *Drosophila in vitro* and in adult abdomens with the addition of ecdysone extracts from *Bombyx mori*. Although ecdysones accelerated pigment deposition and thickening of the discs, no bristles were found, and cuticle formation was not mentioned. Thus, in experiments reported to date, ring glands cause metamorphosis of imaginal discs *in vitro*, but crystalline molting hormone has not yet been found to have this effect. Bodenstein (1943) and Vogt (1944) have shown that *Drosophila* imaginal discs cultured in the abdomen of the adult fail to metamorphose, unless larval ring glands are also transplanted into the host. Since ring glands will cause metamorphosis of imaginal discs both *in vitro* and *in vivo*, whereas crystalline molting hormone has not been shown to cause metamorphosis *in vitro*, it seemed reasonable to test the effects of crystalline molting hormone on imaginal discs cultured *in vivo* before proceeding with further tests *in vitro*. The results to be reported here show that ecdysterone can stimulate complete metamorphosis of *Drosophila* imaginal discs cultured in the adult abdomen. Preliminary results have been reported previously (Postlethwait and Schneiderman, 1968).

MATERIALS AND METHODS

Imaginal first leg discs of mature third instar male and female larvae of an Oregon R stock of *Drosophila melanogaster* were dissected in Ephrussi-Beadle Ringer solution. For most experiments, one of the paired first leg discs from each larva was used as the control, the other was treated with hormone. Each whole disc was transplanted by the Ephrussi and Beadle (1936) technique (see also Ursprung, 1967) into the abdomen of a three-day-old fertilized female adult fly of the same stock. A large needle, about 150 μ in diameter, was employed to reduce damage to the discs, since cut or damaged discs proliferate more than uninjured imaginal discs (Tobler, 1966). The hosts were allowed to recover from the implantation for twenty-four hours, and then were injected with 0.2 μl of either hormone or control solution. In some experiments the injections were repeated on succeeding days. Although only four days are required to complete metamorphosis *in situ,* Bodenstein (1943) found that leg discs required at least nine days to metamorphose when they were cultured in adult hosts in which ring glands had been implanted. We chose to wait eleven days before retrieving the discs in our experiments.

---

[1] Present address: Department of Organismic Biology, University of California, Irvine, California 92664.

Solutions of ecdysterone (20-hydroxyecdysone, iso-inokosterone) obtained from Rohto Pharmaceutical Company, Osaka, Japan, or cyasterone, generously supplied by Professor Carroll Williams, were made up in 10% ethanol in Ringer. Control animals received 10% ethanol in Ringer without hormone. The mortality rates for control and experimental animals were low and approximately equal. More than 170 hormone-treated implants and 70 control discs were recovered.

The imaginal leg disc consists of a columnar epithelium covered by the peripodial membrane, forming a cellular sac (Auerbach, 1936). The surface of the cells which secretes the cuticle faces the lumen of the sac. During normal pupation, the disc everts: the columnar epithelium enlarges by changes in cell shape (Poodry and Schneiderman, in preparation), and pushes through the peripodial sac so that the chitogenous surface comes to lie on the outside of the anlage. A thin, transparent pupal cuticle is secreted, followed by a melanized, bristle-bearing adult cuticle. These phenomena were examined in the experimental material. Eversion was observed directly in freshly-dissected experimental discs. However, in some experiments, eversion was obscured by subsequent changes. In these cases, a convenient and reliable method of determining relative volumes of imaginal discs was to measure the largest dimension of an unfixed disc in three mutually perpendicular planes with an optical micrometer, and then compute the volume. To allow comparison with the *in situ* situation, the data presented in the tables give the size of the discs relative to the size of the leg disc from the mature third instar larva. The pupal and adult cuticles were observed in Gomori-stained whole mounts (Melander and Wingstrand, 1953), and in sections stained with Mallory's triple stain.

RESULTS

Series I: Single and multiple doses of ecdysterone

Preliminary experiments employed single doses of ecdysterone at final concentrations of 0.01 to 100 μg/g host. The results revealed only a slight stimulation of disc enlargement and no cuticle secretion. Since Bodenstein (1943) found that transplanted ring glands continuously secrete hormone, since the prothoracic gland is necessary for several days for metamorphosis of silk worm pupae (Williams, 1952), and since ecdysterone is readily inactivated by some insects (Ohtaki, Milkman, and Williams, 1968), it seemed likely that a sustained supply of ecdysterone might be necessary for metamorphosis. Therefore in Series I, ecdysterone was injected in multiple as well as single doses into adult abdomens containing discs. The total amount of ecdysterone injected in both cases was equal. The discs were transplanted into female hosts on day one. About twenty-four hours later, all of the experimental and control animals were injected with ecdysterone. One experimental group received a total dose of 7.2 μg/g, and a second group, a total dose of 720 μg/g. The results are shown in Table I. Although the total amount delivered in single- and multiple-dose experiments was the same, discs receiving the total dose in instalments enlarged more and secreted cuticle more often than did discs receiving the same amount of hormone in one dose. Apparently, ecdysterone is necessary as a sustained stimulus for metamorphosis, and not merely as a trigger for development. No bristles, hairs or sensillae were formed in Series I.

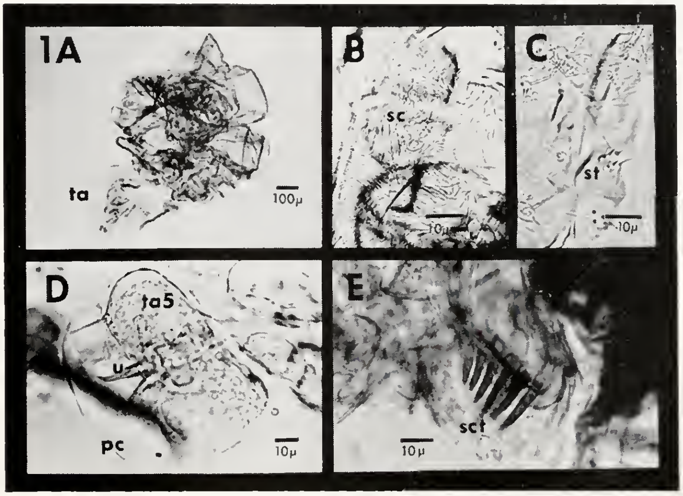


FIGURE 1. Adult structures produced by imaginal leg discs induced to metamorphose by ecdysterone; (A) A metamorphosed leg, note everted tarsus; (B) Sensilla campaniformia of the femur; (C) Sensilla trichodea of the coxa; (D) Claw organ, note pupal cuticle enveloping the tarsus; (E) Sexcomb teeth, typical of the male basitarsus. Abbreviations: pc, pupal cuticle; sc, sensilla campaniformia; sct, sex comb teeth; st, sensilla trichodea; ta, tarsus; ta5, fifth tarsal segment; u, unguis.

Series II: Cyasterone

Cyasterone is an analogue of ecdysone extracted from plants which appears to be more potent than ecdysterone in certain systems (Williams, 1968). Table II shows that a single dose of cyasterone stimulated a greater increase in size and a higher frequency of cuticle secretion than an equivalent dose of ecdysterone. The discs treated with one 720 μg/g dose of cyasterone were larger than discs treated with one 720 μg/g dose of ecdysterone, but were about equal in size to those receiving six injections of 120 μg/g ecdysterone. Whole mounts and sections of these implants showed that a single dose of cyasterone was also more effective in promoting cuticle secretion than a single dose of ecdysterone. Cyasterone appears to be either more stable or intrinsically more potent than ecdysterone.

TABLE I

Series I. Single and multiple doses of ecdysterone

	Mature discs	Total dose of				
		0 μg/g Control	7.2 μg/g		720 μg/g	
			One injection of 7.2 μg/g	Six injections of 1.2 μg/g	One injection of 720 μg/g	Six injections of 120 μg/g
Number of discs	98	63	17	24	16	15
Relative size	1.0	1.2	1.2*	2.4	2.0	2.9
% with cuticle	0	3	12	33	6	60

* Not significantly different from controls, $P = 0.05$.

TABLE II

Series II. Single injections of cyasterone and ecdysterone

	Total dose of			
	1.2 μg/g		720 μg/g	
	Ecdysterone	Cyasterone	Ecdysterone	Cyasterone
Number of discs	6	5	16	8
Relative size	1.0*	2.0	2.0	2.9
% with cuticle	0	20	6	25

* Not significantly different from controls, $p = 0.05$.

Series III: Massive doses of ecdysterone

The experiments reported above demonstrate that repeated doses of ecdysterone, or a single large dose of cyasterone induce partial metamorphosis. Since ecdysterone was readily available, we decided to employ massive doses of ecdysterone (1200 μg/g) delivered once, twice, or three times over the course of twelve days. The results are shown in Table III. Although the final size of these discs was smaller than those in Series I, which had received 720 μg/g ecdysterone in six equal installments, or Series II, which had received one 720 μg/g dose of cyasterone.

TABLE III

Series III. Single and multiple injections of large doses of ecdysterone

	Control	Number of treatments with 1200 μg/g ecdysterone		
		1	2	3
Number of discs	63	4	11	18
Relative size	1.2	2.0	2.5	2.4
% with cuticle	3	100	100	100
% with bristles	0	50	100	100

the discs underwent complete metamorphosis, producing a pupal cuticle, followed by an adult cuticle. All of the normal imaginal structures were formed, including claws, sex combs, sensilla trichodea, sensilla campaniformia, and tibial and tarsal transverse bristle rows (Fig. 1). These appeared to be only two differences between the artificially-metamorphosed adult structures and the corresponding structures *in situ*. First, pigmentation of bristles and cuticle in the experimental situation was irregular, though extensive. Occasionally, bristles in addition to those of the tarsal transverse rows (very pale *in situ*) were unpigmented. Second, the number of bristles formed was often less than in normal imaginal structures. For example, most experimental preparations had only five or six sex comb teeth, whereas the normal male has ten to twelve (Hannah-Alava and Stern, 1957). In one case transdetermination was observed, and both leg and wing structures were found.

Series IV: Eversion

When an imaginal disc is transplanted into a larva, and metamorphoses with the host, it usually fails to evert, but forms a hollow ball with bristles and cuticle secreted on the inside. However, the artificially metamorphosed implants in Series III everted, as judged by the exterior orientation of the bristles. This process was examined in Series IV. Leg discs were injected into adult females, and twenty-four hours later the hosts received a single injection of ecdysterone (either 120 μg/g or 1200 μg/g). Twenty-four hours later, the discs were retrieved and examined. The results are recorded in Table IV, and in Figure 2. Ecdysterone clearly stimulated leg disc eversion, and larger amounts of hormone gave greater stimulation. Table IV shows that in the first twenty-four hours after hormone injection, the discs enlarged greatly. When compared to discs cultured for longer periods (Series I and II), it is seen that the disc enlarges most in the first day. Thus, most of the increase in size observed in Series I and II is probably a result of disc eversion.

TABLE IV

Eversion induced by one dose of 1200 μg/g ecdysterone

	Mature discs	Control	1200 μg/g ecdysterone
Number of discs not everted	22	12	1
Number of discs everted	0	0	6
Relative size	1.0	1.2	2.6

DISCUSSION

The results show that ecdysone analogues can induce complete metamorphosis of *Drosophila* leg discs cultured in the abdomen of adult female flies. A single dose of hormone was not as effective in causing metamorphosis as the same amount delivered in installments. Ohtaki *et al.* (1968) have shown that α-ecdysone is inactivated rapidly in mature larvae of *Sarcophaga,* and Karlson and Bode (1969)

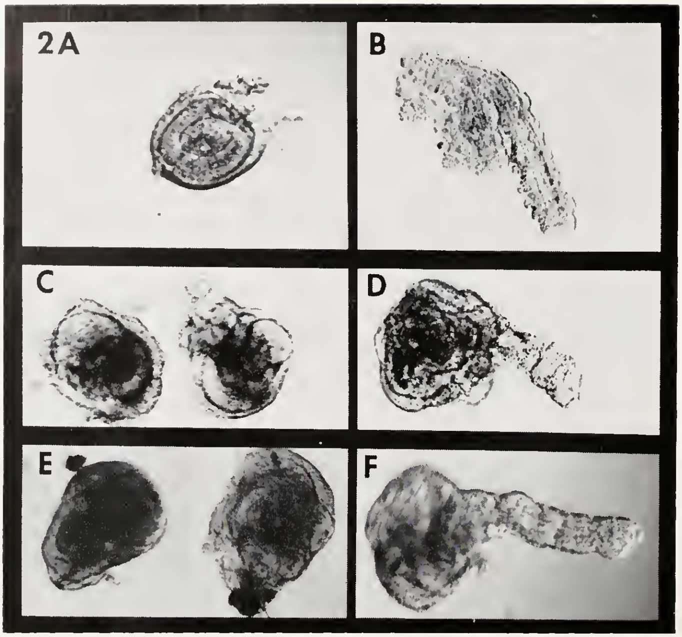


FIGURE 2. Eversion of the imaginal leg disc induced by ecdysterone; (A) Ninety-six hr. imaginal leg disc; (B) Five-hr pupal disc, note eversion; (C) Control discs, 24 hrs after treatment with control solution; (D) Leg disc 24 hrs after treatment with 120 μg/g ecdysterone; (E) Control disc 24 hrs after treatment with control solution; (F) Leg disc 24 hrs after treatment with 1200 μg/g ecdysterone (photograph (B) was taken by C. A. Poodry).

have found that in *Calliphora* its inactivation is due to an enzyme system present in the fat body. Since ecdysterone is probably also degraded in adult *Drosophila*, and repeated small doses are more effective in inducing metamorphosis than single large doses, we conclude that ecdysterone is necessary as a sustained stimulus, and not merely as a trigger to metamorphosis.

In silkmoths, a high dose (10 μg/g) of ecdysterone causes pupal-adult intermediates (Kobayashi, Takemoto, Ogawa and Nishimoto, 1967; Williams, 1968; Madhavan and Schneiderman, personal communication). Diptera, however, such as *Musca* larvae treated with a high dose of ecdysterone (300 μg/g) (Kobayashi, *et al.*, 1967) and *Drosophila* imaginal discs treated with a very high dose of ecdysterone (3600 μg/g) show no such abnormalities. This may be due to rapid inactivation of ecdysterone in Diptera (Ohtaki *et al.*, 1968), or a difference in threshold response to the hormone.

The adult corpus allatum of flies continues to secrete juvenile hormone (Wigglesworth, 1954), which probably is the same factor as the yolk-forming hormone (Wigglesworth, 1948). Traditional explanations of hormonal control of insect development (Schneiderman and Gilbert, 1964) would predict that a given low level of juvenile hormone might permit deposition of pupal cuticle, and the absence of juvenile hormone would allow adult cuticle to be formed. The present experiments show, however, that the presence of juvenile hormone in the adult female host can allow successive production of pupal cuticle and adult cuticle. Perhaps the juvenile hormone titer is too low to exert an effect, or the disc cells have already been determined to secret a pupal cuticle followed by adult structures. Although juvenile hormone has been shown to exert a juvenilizing effect in the blowfly (Srivastava and Gilbert, 1969), the usual juvenile hormone effects are not found in *Drosophila*, even *in situ* (Bryant and Sang, 1968). It would be interesting to examine the effects of juvenile hormone in this system in view of the difficulty of demonstrating juvenile hormone effects in *Drosophila*.

The question arises whether the increase in size of ecdysterone-treated discs is due to cell division. Nearly all of the enlargement can be accounted for by the process of eversion. Poodry and Schneiderman (in preparation) have suggested that changes in cell size and shape, rather than changes in cell number, are primarily responsible for eversion *in situ*. Imaginal discs of *Drosophila* (Enzmann and Haskins, 1938), *Pieris brassicae* (Eassa, 1953), Saturniid silk moths (Krishnakumaran, Berry, Oberlander and Schneiderman, 1967; Patel and Madhavan, 1969) undergo increase in cell number, DNA synthesis, or increase in weight continuously, and independent of the molt cycle. In addition, ecdysterone does not cause DNA synthesis in *Galleria* wing discs *in vitro*, although ecdysone does (Oberlander, 1969). Thus, it is likely that in our experiments and perhaps *in situ*, imaginal discs do not respond to molting hormone by increased cell division except in the case of regeneration (Madhavan and Schneiderman, 1969).

Hadorn (1966) and his colleagues have performed extensive and elegant experiments on the stability of the determined state. The technique involves *in vivo* culture of *Drosophila* imaginal discs in the adult abdomen with periodic tests of the developmental potential of the cell by transplantation of the cultured imaginal disc fragments into larvae, where they metamorphose simultaneously with the host. This metamorphosis test has several limitations: (1) larvae tolerate only small needles and hence only relatively small imaginal disc fragments can be assayed. (2) To obtain a large amount of growth before metamorphosis, it is necessary to transplant the tissue into very young larvae, which are much more difficult to inject than adults. (3) Cutting a large fragment obtained after growth in an adult (in order to get pieces small enough to transplant into larvae) kills cells, distorts the pattern of bristles, and interposes an extra step in the experimental protocol. By employing analogues of ecdysone, the metamorphosis test can be simplified, by using adults instead of larvae, thus permitting metamorphosis of very large fragments. This promises to simplify investigation of certain problems of *Drosophila* development. The results also suggest that unless some other tissue is an intermediary for ecdysone action, *in vitro* metamorphosis of imaginal discs induced by ecdysterone should be possible, a problem currently under investigation.

SUMMARY

The effects of molting hormone on the development of imaginal discs of *Drosophila melanogaster* were investigated. Imaginal leg discs from mature larvae were transplanted into the abdomens of fertilized adult female flies. The hosts were injected with a solution of a phytoecdysone—ecdysterone or cyasterone. The results showed: (1) Large doses of ecdysterone (over 1200 $\mu g/g = 1.2\ \mu g$/fly) caused complete metamorphosis of the transplanted imaginal disc, including eversion

of the disc, formation of a pupal cuticle, and formation of adult cuticle with bristles, hairs and sensilla arranged in normal orientation. (2) Ecdysterone was necessary as a sustained stimulus, and did not act merely as a trigger to metamorphosis. (3) Cyasterone was a more potent molting hormone than ecdysterone. The technique promises to simplify investigation of pattern formation and other developmental problems in *Drosophila.*

LITERATURE CITED

Auerbach, C., 1936. The development of the legs, wings and halteres in wild type and some mutant strains of *Drosophila melanogaster. Trans. Roy. Soc. Edinburgh,* **58**: 787–815.

Bodenstein, D., 1943. Hormones and tissue competence in the development of *Drosophila. Biol. Bull.,* **84**: 34–58.

Bryant, P., and J. Sang, 1968. Drosophila: lethal derangements of metamorphosis and modifications of gene expression caused by juvenile hormone mimics. *Nature,* **220**: 393–394.

Burdette, W., E. W. Hanley and J. Grosch, 1968. The effect of ecdysones on the maintenance and development of ocular imaginal discs *in vitro. Texas Reports on Biology and Medicine,* **26**: 173–180.

Day, M., and T. D. C. Grace, 1959. Culture of insect tissues. *Ann. Rev. Entomol.,* **4**: 17–38.

Eassa, Y., 1953. The development of imaginal buds in the head of *Pieris brassicae* (Lepidoptera) *Trans. Roy. Ent. Soc. London,* **104**: 39–50.

Enzmann, E., and C. Haskins, 1938. The development of the imaginal eye in the larva of *Drosophila melanogaster. J. Morphol.,* **63**: 63–73.

Ephrussi, B., and G. Beadle, 1936. A technique of transplantation for *Drosophila. Amer. Natur.,* **70**: 218–225.

Gehring, W., 1968. The stability of the determined state in cultures of imaginal discs in *Drosophila,* pp. 136–154. *In:* H. Ursprung, Ed., *Results and Problems in Cell Differentiation, Volume 1, The Stability of the Differentiated State.* Springer-Verlag, New York.

Gottschewski, G., 1960. Morphogenetische untersuchungen an *in vitro* wachsenden Augenanlagen von *Drosophila melanogaster. Wilhelm Roux' Arch. Entwickslungsmech. Organismen.,* **152**: 204–229.

Hadorn, E., 1966. Konstanz, Wechsel und Typus der Determination und Differenzierung in Zellen aus Männlichen Genitalanlagen von *Drosophila melanogaster* nach Dauerkultur *in vivo. Dev. Biol.,* **13**: 424–509.

Hannah-Alava, A., and C. Stern, 1957. The sexcombs in males and intersexes of *Drosophila melanogaster. J. Exp. Zool.,* **134**: 533–556.

Karlson, D., and C. Bode, 1969. Die Inaktivierung des Ecdysons bei der Schmeissfliege *Calliphora erythrocephala* Meigen. *J. Insect Physiol.,* **15**: 111–118.

Kobayashi, M., T. Takemoto, S. Ogawa and N. Nishimoto, 1967. The molting hormone activity of ecdysterone and inokosterone isolated from *Achyranthis radix. J. Insect Physiol.,* **13**: 1395–1400.

Krishnakumaran, A., S. Berry, H. Oberlander and H. Schneiderman, 1967. Nucleic acid synthesis during insect development. II. Control of DNA synthesis in the Cecropia silkworm and other Saturniid moths. *J. Insect Physiol.,* **13**: 1–57.

Kuroda, Y., and K. Yamaguchi, 1956. The effects of the cephalic complex upon the eye discs of *Drosophila melanogaster. Jap. J. Genetics,* **31**: 98–103.

Madhavan, K., and H. Schneiderman, 1969. Hormonal control of imaginal disc regeneration in *Galleria mellonella* (Lepidoptera). *Biol. Bull.,* **137**: 321–331.

Melander, Y., and K. Wingstrand, 1953. Gomori's hematoxylin as a chromosome stain. *Stain Technol.,* **28**: 217–223.

Oberlander, H., 1969. Effects of ecdysone, ecdysterone, and inokosterone on the *in vitro* initiation of metamorphosis of wing discs of *Galleria mellonella. J. Insect Physiol.,* **15**: 297–304.

Oberlander, H., and L. Fulco, 1967. Growth and partial metamorphosis of imaginal discs of the greater wax moth *Galleria mellonella, in vitro. Nature,* **216**: 1140–1141.

OHTAKI, T., R. MILKMAN AND C. WILLIAMS, 1968. Dynamics of ecdysone secretion and action in the fleshfly *Sarcophaga peregrina*. *Biol. Bull.*, **135**: 322–334.

PATEL, N., AND K. MADHAVAN, 1969. Effects of hormones on RNA and protein synthesis in the imaginal wing discs of the ricini silkworm. *J. Insect Physiol.*, **15**: 2141–2149.

POSTLETHWAIT, J., AND H. SCHNEIDERMAN, 1968. Effects of an ecdysone on growth and cuticle formation of *Drosophila* imaginal discs cultured *in vivo*. *Biol. Bull.*, **135**: 431–432.

SCHNEIDER, I., 1964. Differentiation of larval *Drosophila* eye-antennal discs in vitro. *J. Exp. Zool.*, **156**: 91–104.

SCHNEIDER, I., 1966. Histology of larval eye-antennal disc and cephalic ganglia of *Drosophila* cultured *in vitro*. *J. Embryol. Exp. Morphol.*, **15**: 271–279.

SCHNEIDERMAN, H., AND L. GILBERT, 1964. Control of growth and development in insects. *Science*, **143**: 325–333.

SRIVISTAVA, U., AND L. GILBERT, 1969. The influence of juvenile hormone on the metamorphosis of *Sarcophaga bullata*. *J. Insect Physiol.*, **15**: 177–189.

TOBLER, H., 1966. Zellspezifische Determination und Beziehung zwischen Proliferation und Transdetermination in Bein- und Flügel primordien von *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.*, **16**: 609–633.

URSPRUNG, H., 1967. *In vitro* culture of Drosophila imaginal discs, pp. 485–492. *In:* F. Wilt and N. Wessels, Ed., *Methods in Developmental Biology*. Thomas Y. Crowell Company, New York.

VOGT, M., 1944. Induktion von Metamorphoseprozessen durch implantierte Ringdrüsen bei Drosophila. *Wilhelm Roux' Arch. Entwicklungsmech. Organismen.*, **142**: 131–182.

WIGGLESWORTH, V., 1948. The function of the corpus allatum in *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol.*, **25**: 1–15.

WIGGLESWORTH, V., 1954. Secretion of juvenile hormone by the corpus allatum of *Calliphora*. *Nature*, **174**: 556.

WILLIAMS, C., 1952. Morphogenesis and the metamorphosis of insects. *The Harvey Lectures*, **47**: 126–155.

WILLIAMS, C., 1968. Ecdysone and ecdysone-analogues: their assay and action on diapausing pupae of the cynthia silkworm. *Biol. Bull.*, **134**: 344–355.

Reference: *Biol. Bull.*, **138**: 56–65. (February, 1970)

REPRODUCTIVE PHYSIOLOGICAL VARIATION IN LATITUDINALLY SEPARATED POPULATIONS OF THE BAY SCALLOP, *AEQUIPECTEN IRRADIANS* LAMARCK[1]

A. N. SASTRY

Graduate School of Oceanography, University of Rhode Island, Kingston, Rhode Island 02881

Populations of marine animals exposed to different environments within their geographical range may vary in physiological properties. Bullock (1955), Prosser (1955) and Vernberg (1962) reviewed the earlier literature on this subject and discussed the importance of physiological differences in the study of distribution, acclimation and speciation. In many of these studies physiological differences have been compared by measuring rate processes such as oxygen consumption and heart beat. Population differences in resistance to environmental extremes have also been demonstrated for marine animals (Vernberg, 1962). More recently, progress has been made in understanding the mechanisms of temperature acclimation (Prosser, 1967; Troshin, 1967).

TABLE I

The mean size and the mean body weight of monthly samples of scallops analyzed for determination of the reproductive activity

Month	Woods Hole, Massachusetts		Beaufort, North Carolina	
	Size mm mean ± S.D.	Body weight g mean ± S.D.	Size mm mean ± S.D.	Body weight g mean ± S.D.
1965				
January	61.33 ± 3.57	16.91 ± 5.23	71.01 ± 4.52	38.87 ± 7.60
February	61.55 ± 3.40	18.68 ± 5.69	30.00 ± 6.22	2.62 ± 1.97
March	42.71 ± 3.44	5.44 ± 1.33	27.50 ± 3.42	1.82 ± 1.35
April	43.33 ± 1.99	4.82 ± 1.68	26.35 ± 6.89	1.75 ± 1.45
May	53.71 ± 8.83	9.59 ± 5.99	31.43 ± 0.44	1.90 ± 0.17
June	57.97 ± 11.86	14.05 ± 9.39	33.47 ± 4.36	3.57 ± 1.41
July	49.28 ± 2.39	8.22 ± 0.81	49.35 ± 5.24	10.00 ± 3.04
August	52.77 ± 5.22	8.51 ± 1.96	62.18 ± 3.54	22.23 ± 3.92
September	59.11 ± 6.02	11.32 ± 2.87	62.41 ± 4.22	22.05 ± 3.55
October	56.09 ± 3.42	11.62 ± 2.77	62.19 ± 7.05	20.41 ± 1.97
November	62.73 ± 5.56	25.44 ± 6.20	56.15 ± 4.60	15.25 ± 2.89
December	49.53 ± 4.05	8.06 ± 2.25	51.03 ± 5.71	10.45 ± 3.05

The geographical range of a species is regulated by the environmental limits for its survival, functioning and reproduction (Hutchins, 1947; Spark, 1957). Varying adaptive capacities of populations exposed to different environments may serve as a primary mechanism in speciation (Prosser, 1960; Mayr, 1963). The reproductive physiological response of populations exposed to different environments within the overall range of a species has been examined for few marine animals. Consequently, the adaptive mechanisms for reproduction in different environments and their role in the speciation process are not well understood.

---

[1] This study was supported by National Science Foundation Grant GB-1356. The work was done at the Duke University Marine Laboratory, Beaufort, North Carolina.

The bay scallop, *Aequipecten irradians* Lamarck, has a wide distribution along the Atlantic and Gulf Coasts of the United States, resulting in many separate populations exposed to different environments (Abbott, 1954; Schroeder, 1966). Populations of this species in different parts of the range vary in the timing of the spawning period (Sastry, 1963). The present study examines the annual reproductive activity of populations of scallops occurring in different temperature environments. The effects of food and temperature on the reproductive response of scallops have been investigated in a series of laboratory experiments, the results of which were reported earlier by Sastry (1966a, 1968).

MATERIALS AND METHODS

Populations of *A. irradians* examined in the present study were collected in the Falmouth area of Nantucket Sound near Woods Hole, Massachusetts and off Beau-

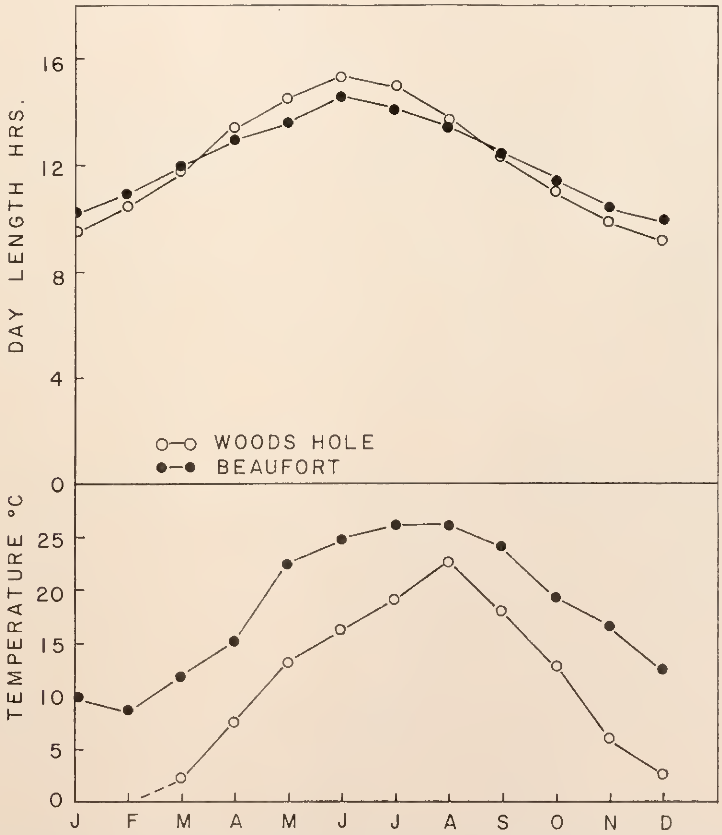


FIGURE 1. Monthly mean sea water temperature and the average day-length in the geographical locations where the scallops were collected.

fort, North Carolina (Table I). The two populations are exposed to significantly different environmental temperatures throughout the year (Fig. 1). Monthly samples collected near Woods Hole were flown live in chilled containers to Beaufort for analysis. The Beaufort scallops were collected near the Duke University Marine Laboratory.

The population samples were analyzed to determine the monthly gonad index (gonad weight/body weight × 100), digestive gland index (digestive gland weight/body weight × 100) and the stage in gametogenesis according to the methods

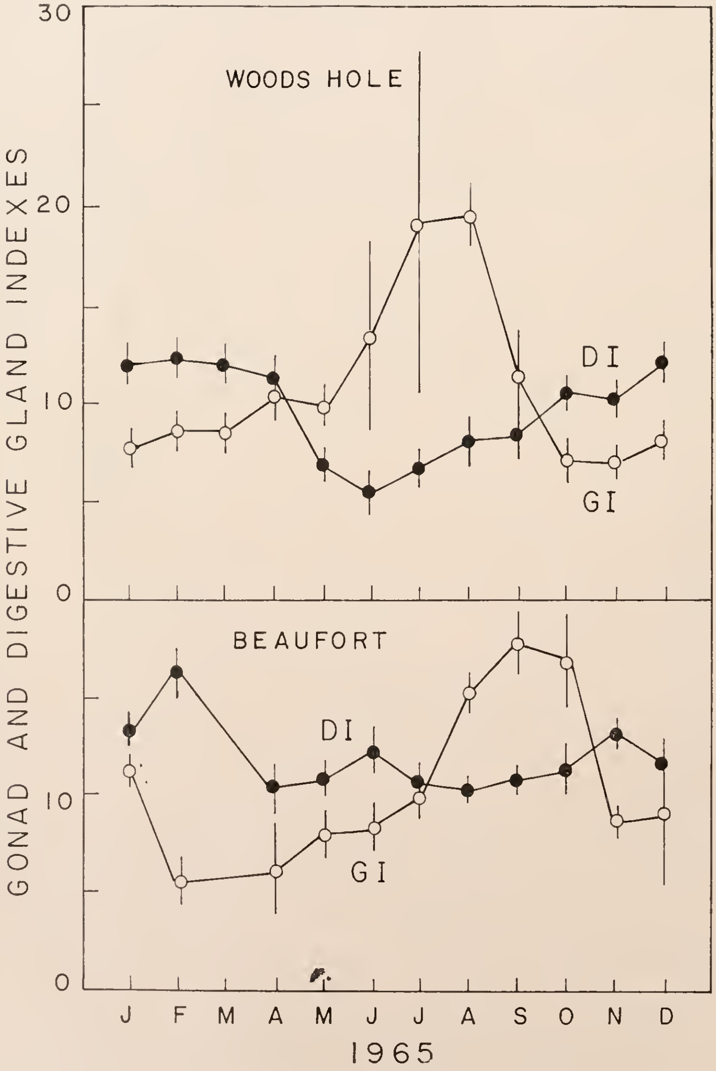


FIGURE 2. Observed monthly mean gonad and digestive gland index values for the populations of scallops from Woods Hole, Massachusetts and Beaufort, North Carolina. Vertical lines show the 95% confidence intervals from the mean.

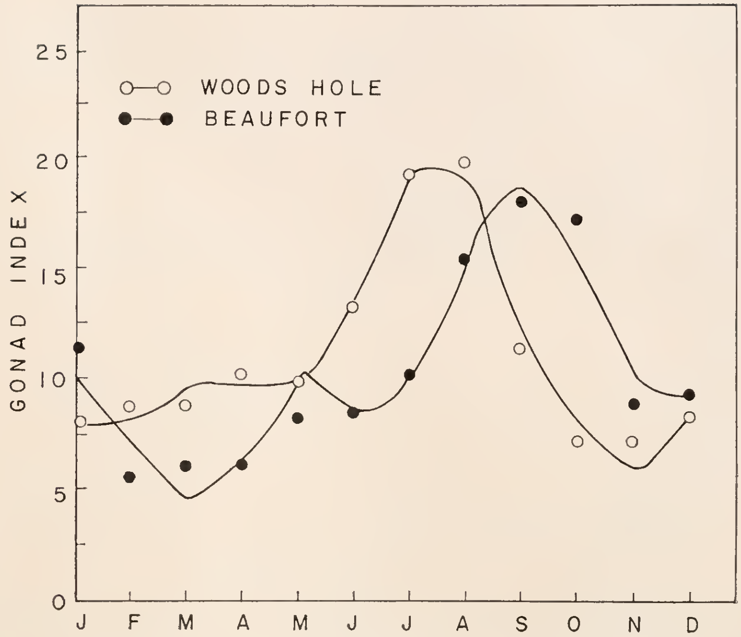


FIGURE 3. Monthly mean gonad index values for the populations of scallops fitted with the three-term Fourier curves show a cyclical trend in gonad activity response within the twelve month period.

described by Giese (1959) and Sastry (1966a). The observed monthly gonad index values were fitted with an annual trend line determined by the method of periodic regression analysis described by Bliss (1958).

OBSERVATIONS

Annual gonad cycle

The monthly mean gonad index values for the two populations of scallops are shown in Figure 2. The three-term Fourier curves fitted to the observed data clearly show the cyclical gonad activity of the populations during the year (Fig. 3). The annual gonad cycle of the scallops includes vegetative, growth and gametogenesis, maturation, spawning, and resting periods (Sastry, 1966a), but these stages are reached at different times in the two populations. Gonad growth takes place from April through July at Woods Hole, from June through September at Beaufort. The duration of gonad growth activity is approximately the same for both populations. The gonad index seems to decrease with the onset of spawning (Sastry, 1966a). The Woods Hole population has completed spawning by September, the time of the maximum gonad growth at Beaufort.

Relationship between the changes in gonad and digestive gland indexes

In the annual cycle, gonad growth seems to occur by an accumulation of nutrient reserves in the tissue which are apparently utilized by the developing gametes for

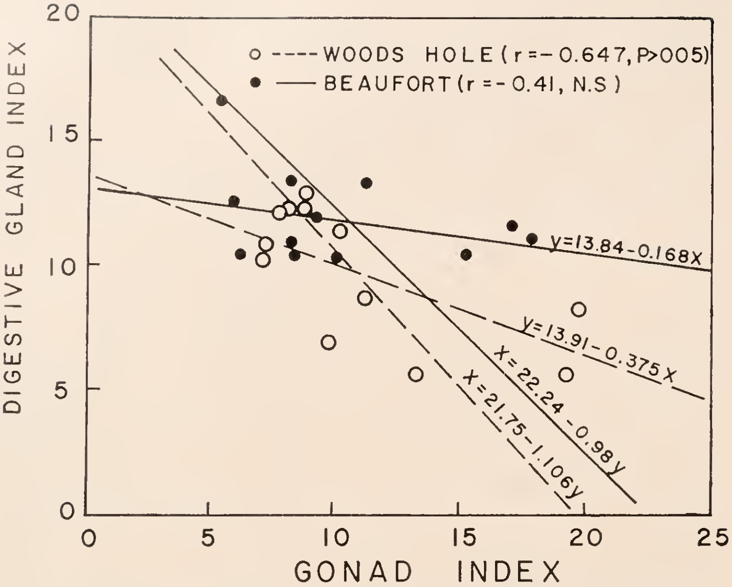


FIGURE 4. Correlation between monthly mean gonad index and the digestive gland index of the populations of scallops.

synthesis of various biochemical constituents (Marshall and Orr, 1961; Giese, 1959; Barnes, Barnes and Finlayson, 1963; Sastry, 1966a, 1968). The changes in the monthly gonad and digestive gland indexes in some marine invertebrates show a reciprocal relationship suggesting the influence of food on gonad growth (Giese, 1959; Sastry, 1966a, 1968). The digestive gland index of the scallop populations is at a relatively higher level than the gonad index during the vegetative and resting periods (Fig. 2). To determine the significance of the relationship, correlation coefficients were computed. Both populations showed a negative correlation, but this was significant at the 5% probability level only for the population at Woods Hole (Fig. 4). The changes in the monthly digestive gland index probably indicates the amounts of nutrient reserves in the tissue which vary with gonad growth activity and seasonal changes in the environment. In a number of laboratory experiments, it has been shown that scallops in the resting stage require abundant food and exposure to minimum temperature to begin gonad growth (Sastry, 1968). The nutrient reserves present in the digestive gland, however, are obviously not adequate to sustain gonad growth. Continuing growth is apparently related to periods of abundant food production.

Gametogenesis

The predominant stages in gametogenesis observed in the two populations of scallops are given in Table II. Similar stages in gametogenesis appear in both populations during different months of the year. The Woods Hole population develops oogonia by March, and the oocytes begin the synchronous growth phase during April. Vitellogenesis is underway in May, and by late June the oocytes have begun to mature. Fertilizable eggs are present from the middle of July through August. The gonads are neutral from September to December.

The Beaufort population also develops a few oogonia by March and the oocytes are in the synchronous growth phase in April. These oocytes complete vitellogenesis during May, but they disintegrate before becoming fertilizable eggs. However, at this time the population again develops oogonia, in larger numbers than before. The oocytes developed by June begin the synchronous growth phase. Vitellogenesis begins in July, and the oocytes become fertilizable eggs by late September. The population is in a mature reproductive state from then until the middle of November.

TABLE II

The predominant stages in gametogenesis observed in the populations of scallops during different months of the year*

Stage in gametogenesis	Woods Hole, Massachusetts	Beaufort, North Carolina
Oogenesis		
Primary germ cells	January–February	January–April
Oogonia	March	May
Synchronous oöcyte growth	April–May	June–July
Vitellogenesis	May–June	July–September
Fertilizable eggs	July–September	September–November
Spermatogenesis		
Spermatogonia	January–March	January–April
Spermatocytes	April–May	May–June
Spermatids	June	July–August
Spermatozoa	July–September	August–November
Neutral gonads	September–December	Late November–December

* A detailed description of the stages in gametogenesis of scallops is given by Sastry (1966a).

The monthly mean oocyte diameter is shown in Figure 5. The monthly oocyte growth rates for the two populations were compared by the method of analysis of covariance (Snedecor and Cochran, 1967). The oocyte growth rate in the Woods Hole population ($Y = 9.92\,X - 3.31$) was higher than in the Beaufort population ($Y = 7.0\,X - 3.9$).

Statistical analysis of variation in the annual reproductive cycle of the populations

The three-term Fourier curves fitted to show the cyclical gonad activity are described by the following periodic regression equations:

$Y = 11.03 - 4.6631\,u_1 + 0.03927\,v_1 + 2.6816\,u_2 + 1.51242\,v_2 - 1.003\,u_3 - 1.4883\,v_3$ (Woods Hole); and

$Y = 10.36 - 2.0101\,u_1 - 4.5021\,v_1 - 0.500\,u_2 + 1.2925\,v_2 + 2.1416\,u_3 + 0.0533\,v_3$ (Beaufort).

In these equations, the first term describes the fundamental cycle (sine curve) for the year. Addition of the remaining two terms recovers the curves fitted for the observed data. The variation in the regression coefficients in the first term of

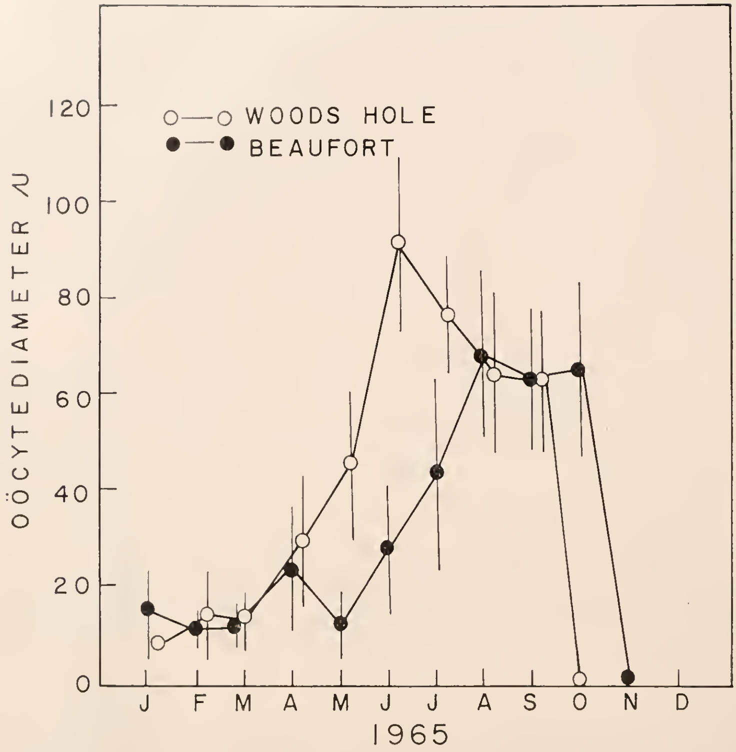


FIGURE 5. Observed monthly mean oocyte diameter for the populations of scallops. Vertical lines show 95% confidence intervals from the mean.

the two equations indicates a possible difference in amplitude, in phase angle (time of maximum) or both (Bliss, 1958). The estimated amplitude of gonad response for the two populations is approximately the same (Woods Hole, 9.32; Beaufort, 9.80). However, the maximum amplitude occurs about June 25 at Woods Hole and August 19 at Beaufort. This displacement is probably the most significant difference between the annual gonad activity cycles of the two populations. The difference is apparently due to the two populations interacting in the same way with different conditions of temperature and food abundance.

DISCUSSION

Physiological variation in populations of a species exposed to different environments could be either a phenotypic response of a single genotype or could be truly genetic (Prosser, 1955). Genetic divergence of populations may bring differences in adaptive capacities and thus serve as a mechanism in species differentiation. Comparison of physiological responses of some geographically separated populations of marine animals has shown variation in adaptive capacity, but in many cases the difference has been phenotypic.

The processes examined in the earlier studies are directly related to the survival of adult organisms in the different environments, but reproductive physiological activity is concerned with species continuity. Variation in reproductive activity may therefore be adaptive for continuity of populations in geographically separated areas. It may thus become significant in evolutionary divergence.

The reproductive physiological process is a cyclical activity that may be repeated on an annual, semi-annual or continuous basis (Giese, 1959). The pattern of annual reproductive activity may be considered as a genetically controlled response to the environment. This is seen clearly in some marine invertebrates in temperate zones. Successive events in the reproductive cycle correlate with differences in the environment that develop during the year. Thus, a succession of environmental factors influences the events in the annual reproductive cycle. It is possible that the timing of reproductive events may have to be coordinated with the seasonal changes in the environment to exhibit the characteristic cycle of annual gonad activity. The temperatures corresponding to the successive events in the reproductive cycle are lower at Wood Hole than at Beaufort (Figs. 1 and 2). Ryther and Yentsch (1958) and Yentsch (1963) reported that phytoplankton production reached peak levels during the winter and spring in Northern locations. At Beaufort, the phytoplankton production peaks during the summer months (Williams and Murdoch, 1966). Thus, the displacement of reproductive events of the populations may have been favored by selection as an adaptive response to geographical differences in the period of abundant food production and temperatures.

To determine whether variation in annual reproductive activity of scallops is genetic or environmental, acclimation tests will have to be performed (Prosser, 1955; Sastry, 1966b and Sastry, unpublished observations). The basis of variation among geographically separated populations is not clear for most marine invertebrates. However, it has been suggested that the breeding period of a species occurs at characteristic temperatures which remain a constant throughout the geographical range (Orton, 1920). Runnstrom (1927) suggested that the breeding period of widely distributed marine animals is earlier in the southern part of their range, but at about the same temperatures as in the North. Although, temperature affects reproduction, other environmental factors appear to interact with it in determining the pattern of annual gonad activity in a given geographical area. It is likely the variation in annual reproductive activity of a species will be the phenotypic response of a single genotype. Alternately, however, the variation may result from genetic divergence of the populations in different parts of the range. In that case the populations would not reciprocally acclimate. Stauber (1950), Loosanoff and Nomejko, (1951), and Korringa (1957) observed that the critical minimum temperatures for spawning of geographically separated populations of *Crassostrea* and *Ostrea* were different and suggested that they might represent physiological races. The populations of scallops in the present study vary in the time of year when successive reproductive events take place and also in the environment for these events. It is possible that the variation in the populations of scallops could have been induced by their coordination of reproductive events with the environment at the whole organism level.

The author would like to thank Dr. Nelson Marshall for critical reading of the manuscript.

SUMMARY

1. The geographically separated populations of the bay scallop *A. irradians* from Woods Hole, Massachusetts, and Beaufort, North Carolina, vary in their timing of reproductive events. These events occur earlier in the year at Woods Hole than at Beaufort.

2. The monthly gonad index values of the populations are fitted with three-term Fourier curves to show the cyclical trend of gonad activity response within the year. Periodic regression analysis of the data indicates that displacement of maximum gonad response is a significant difference between the two populations.

3. The reproductive physiological variation in the populations of scallops may have been favored through selection as an adaptive response to the geographical differences in temperature and time of abundant food production.

4. The variation could be either phenotypic or truly genetic.

LITERATURE CITED

Abbott, R. T., 1954. *American Seashells.* Van Nostrand Co., New York, 541 pp.

Barnes, H., M. Barnes and D. M. Finlayson, 1963. The seasonal changes in body weight, biochemical composition and oxygen uptake of two boreo-arctic barnacles, *Balanus balanoides* and *B. balanus. J. Mar. Biol. Ass. U. K.,* **43**: 185–212.

Bliss, C. I., 1958. Periodic regression in Biology and Climatology *Conn. Agr. Exp. Sta. New Haven Bull.,* **615**: 1–55.

Bullock, T. H., 1955. Compensation for temperature and activity of poikilotherms. *Biol. Rev.,* **30**: 1–22.

Giese, A. C., 1959. Comparative physiology: Annual reproductive cycles of marine invertebrates. *Annu. Rev. Physiol.,* **21**: 547–576.

Hutchins, L. W., 1947. The bases of temperature zonation in geographic distribution. *Ecol. Monogr.,* **17**: 325–335.

Korringa, P., 1957. Water temperature and breeding throughout the geographical range of *Ostrea edulis. Annee. Biol.,* **33**: 1–17.

Loosanoff, V. L., and C. J. Nomejko, 1951. Existence of physiologically different races of oysters, *Crassostrea virginica. Biol. Bull.,* **101**: 151–156.

Marshall, S. M., and A. P. Orr, 1961. Studies on the biology of *Calanus finmarchicus.* XII. The phosphorus cycle: excretion, egg production and autolysis. *J. Mar. Biol. Ass. U. K.,* **41**: 463–488.

Mayr, E., 1963. *Animal Species and Evolution.* Belknap Press, Cambridge, Massachusetts, 799 pp.

Orton, J. H., 1920. Sea temperature, breeding and distribution of marine animals. *J. Mar. Biol. Ass. U. K.,* **12**: 339–366.

Prosser, C. L., 1955. Physiological variation in animals. *Biol. Rev.,* **30**: 229–262.

Prosser, C. L., 1960. Comparative physiology in relation to evolutionary theory, pp. 569–594. *In:* Sol Tax, Ed. *Evolution after Darwin, The Evolution of Life.* University of Chicago Press, Chicago, Illinois.

Prosser, C. L., (Ed.), 1967. *Molecular Mechanisms of Temperature Adaptation.* Symposium American Association Advancement Science, Washington, D. C., 398 pp.

Runnstrom, S., 1927. Über die Thermopathie der Fortpflanzung und Entwicklung mariner Tiere in Bezeihung zu ihrer geographischen Verbreitung. *Bergens. Mus. Arbog,* **2**: 1–67.

Ryther, J. H., and C. S. Yentsch, 1958. Primary production of continental shelf waters off New York. *Limnol. Oceanogr.,* **3**: 327–335.

Sastry, A. N., 1963. Reproduction of the bay scallop, *Aequipecten irradians* Lamarck. Influence of temperature on maturation and spawning. *Biol. Bull.,* **125**: 146–153.

Sastry, A. N., 1966a. Temperature effects in reproduction of the bay scallop, *Aequipecten irradians* Lamarck. *Biol. Bull.,* **130**: 118–134.

Sastry, A. N., 1966b. Variation in reproduction of latitudinally separated populations of two marine invertebrates. *Amer. Zool.,* **5**: 374–375.

Sastry, A. N., 1968. The relationships among food, temperature and gonad development of the bay scallop, *Aequipecten irradians* Lamarck. *Physiol. Zool.,* **41**: 44–53.

Schroeder, E. H., 1966. Average surface water temperatures of the western North Atlantic. *Bull. Mar. Sci.,* **16**: 302–323.

Snedecor, G. W., and W. G. Cochran, 1967. *Statistical Methods.* Iowa State University Press, Ames, Iowa, 593 pp.

Spärk, R., 1967. The importance of metabolism in the distribution of marine animals. *Annee. Biol.,* **33**: 232–239.

Stauber, L. A., 1950. The problem of physiological species with a special reference to oysters and oyster drills. *Ecology,* **31**: 107–118.

Troshin, A. S. (Ed.), 1967. *The Cell and Environmental Temperature.* [International Series of Monographs in Pure and Applied Biology, Vol. 34] Pergamon Press, New York, 462 pp.

Vernberg, F. J., 1962. Comparative physiology: Latitudinal effects on physiological properties of animals. *Annu. Rev. Physiol.,* **25**: 517–546.

Williams, R., and M. B. Murdoch, 1966. Phytoplankton production and chlorophyll concentrations in Beaufort Channel, North Carolina. *Limnol. Oceanogr.,* **2**: 73–82.

Yentsch, C. S., 1963. Primary production, pp. 157–175. *In:* H. Barnes, Ed., *Oceanography and Marine Biology, Annual Review, Vol. 1.* George Allen and Unwin, Ltd., London.

Reference: *Biol. Bull.*, 138: 66–76. (February, 1970)

THE MARINE CERCARIAE OF THE WOODS HOLE MASSACHUSETTS REGION[1]

HORACE W. STUNKARD

The American Museum of Natural History, Central Park West at 79th Street, New York, New York 10024

The study of animal parasites in the Woods Hole region began with the report by Verrill and Smith (1873) on the invertebrate animals of Vineyard Sound and adjacent waters. They stated, page 455, "Large numbers of fishes were examined, both internally and externally, for parasites, and a large collection of such parasites was made. The internal parasites were collected mainly by Dr. Edward Palmer, and will be of great interest when carefully studied and described. As yet, nothing more than a casual examination of them has been made." At the time, life-cycles of marine helminths were quite unknown. However, they noted, page 457, "Most of the species that, in the adult state inhabit fishes, live while young, or in the larval stages, in smaller fishes, or in other animals, upon which the larger fishes feed, and from which they thus derive their parasites."

Professor Verrill continued to be a member of the group associated with Professor Spencer F. Baird, U. S. Commissioner of Fisheries, and was in charge of the summer laboratory located on Little Harbor in Woods Hole. In the summer of 1884, Professor Baird encouraged Edwin Linton, a graduate student with Professor Verrill at Yale and since 1882 an assistant during the summer months at the Fish Commission, to begin the study of the Entozoa of marine fishes. Although a full-time member of the faculty at Washington and Jefferson College, Linton continued his summer researches on the parasites of fishes, describing cestodes, nematodes, trematodes and acanthocephalans. The investigation was continued for the next fifty years and most of the work was done at the laboratory of the Fish Commission, later designated as a Bureau in the U. S. Department of Commerce. The study was concerned chiefly with the adult stages of these worms, their morphology, bionomics, and systematic position. Larval stages of the helminths and the occurrence of protozoan species were recorded occasionally, but the observations were incidental to other interests.

Knowledge of larval trematodes has progressed more rapidly and much farther than that of other marine helminths. Ryder (1884) reported larvae encysted in the skin of cunners, *Tautogolabrus adspersus,* taken at Woods Hole and Cape Breton, Nova Scotia, and suspected that they were trematodes. Linton (1900, 1901) recorded the parasites from cunners and tautogs at Woods Hole and Stafford (1905) named them *Dermocystis ctenolabri.* During the summers of 1909 and 1910, Linton examined invertebrates for larval trematodes and the results were published in 1915. On July 24, 1911, Linton dissected a loon and found 4789 distomes in the intestine; there were various developmental stages and the youngest were so similar to the larvae encysted in the cunner that he (1915a) announced their specific identity. The worms were identified as *Tocotrema lingua* (Creplin). but *Tocotrema* Looss, 1899 is a synonym of *Cryptocotyle* Lühe, 1899. Linton (1915b) reported a furcocercous cercaria with a dorsal fin from sporocysts in the serpulid annelid, *Hydroides dianthus* Verrill, and (1915c) similar sporocysts and cercariae from the scallop, *Pecten irradians*. The latter paper contains, also, the description of sporocysts and tailless cercariae from *Ilyanassa obsoleta* (=*Nassarius obsoletus*). The furcocercous cercaria from *H. dianthus* was studied by Stunkard (1929), who recognized it as a blood-fluke of fishes and named it *Cercaria loossi.* Further observations were made by Martin (1944). The species from *I. obsoleta* was studied by Miller and Northup (1926) and named *Cercariaeum lintoni*. They described five cercarial species from *N. obsoletus*. The first life-cycle to be completed and demonstrated experimentally was that of *Cryptocotyle lingua,* done by Stunkard (1930). Since that time, life-cycles of digenetic trematodes have been studied by Cable, Hunninen, Martin, Rankin, Stunkard, and Uzmann, and the developmental stages of thirty-two species are known. As a rule, the first intermediate host, the mollusk, is more specific than the definitive vertebrate host; the second intermediate host, when present, may be a mere paratenic or transport host. In it, the metacercaria may encyst or not, may fail to develop or grow to almost full-size and attain sexual maturity, a condition known as progenesis. The evolution of the life-history in the Digenea was discussed by Stunkard (1960c).

---

[1] Investigation supported by NSF, GB-8423.

MARINE TREMATODES OF THE WOODS HOLE AREA

Families and species with known life-cycles

ACANTHOCOLPIDAE
- *Stephanostomum dentatum* (Linton, 1901) Linton, 1940.
- *Stephanostomum tenue* (Linton, 1898) Martin, 1938.

CRYPTOGONIMIDAE
- *Siphodera vinaledwardsi* (Linton, 1901) Linton, 1910.

ECHINOSTOMATIDAE
- *Himasthla compacta* Stunkard, 1960.
- *Himasthla littorinae* Stunkard, 1966.
- *Himasthla quissetensis* (Miller and Northup, 1926) Stunkard, 1938.

FELLODISTOMATIDAE
- *Parvatrema borealis* Stunkard and Uzmann, 1958.
- *Proctoeces maculatus* (Looss, 1901) Odhner, 1911.

HEMIURIDAE
- *Lecithaster confusus* Odhner, 1905.

HETEROPHYIDAE
- *Cryptocotyle lingua* (Creplin, 1825) Fischoeder, 1903.

LEPOCREADIIDAE
- *Deropristis inflata* (Molin, 1859) Odhner, 1902.
- *Homalometron pallidum* Stafford, 1904.
- *Lepocreadium setiferoides* (Miller and Northup, 1926) Martin, 1938.
- *Neopechona pyriforme* (Linton, 1900) Stunkard, 1969.

MICROPHALLIDAE

Gynaecotyle nassicola (Cable and Hunninen, 1938) Rankin, 1940.
Levinseniella minuta Price, 1934.
Microphallus limuli Stunkard, 1951.
Microphallus nicolli (Cable and Hunninen, 1938) Baer, 1943.
Microphallus similis (Jägerskiöld, 1900) Baer, 1943.

MONORCHIIDAE

Monorcheides cumingiae (Martin, 1938) Martin, 1940.

NOTOCOTYLIDAE

Notocotylus atlanticus Stunkard, 1966.
Notocotylus minutus Stunkard, 1960.
Paramonostomum alveatum (Mehlis in Creplin, 1846) Lühe, 1909.
Paramonostomum parvum Stunkard and Dunihue, 1931.
Uniserialis breviserialis Stunkard, 1967.

OPECOELIDAE

Opecoeloides manteri (Hunninen and Cable, 1940) Hunninen and Cable, 1941.
Podocotyle atomon (Rudolphi, 1802) Odhner, 1905.

PHILOPHTHALMIDAE

Parorchis acanthus (Nicoll, 1906) Nicoll, 1907.

RENICOLIDAE

Renicola thaidus Stunkard, 1964.

SCHISTOSOMATIDAE

Microbilharzia variglandis (Miller and Northup, 1926) Stunkard and Hinchliffe, 1951.

ZOOGONIDAE

Zoogonoides laevis Linton, 1940.
Zoogonus lasius (Leidy, 1891) Stunkard, 1940.

LARVAL TREMATODES OF THE WOODS HOLE AREA

** Designates publication with figures of larvae*

In *ANACHIS AVARA:*

1. Cercaria of *Neopechona pyriforme.* Distomate, ocellate, trichocercous, cuticula with retrorse spines, excretory vesicle cylindrical, contains concretions and extends anterior to the acetabulum, flame-cell formula 2 [(4 + 4 + 4) + (4 + 4 + 4)], develop in simple rediae with attenuate, curved, tail-like posterior ends; metacercariae unencysted in *Mnemiopsis leidyi* and medusae of *Bougainvillia carolinensis, Gonionemus vertens, Chrysaora quinquecirrha, Pelagia noctulica,* and *Aequoria forskalea;* adults experimentally in scup, *Stenotomus chrysops.* Species described by Linton (1900, 1901) as *Distomum pyriforme* from several species of fish. *(Stunkard, 1969.)

In *BITTIUM ALTERNATUM:*

1. Cercaria of *Microphallus nicolli.* (Described as *Spelotrema nicolli* Cable and Hunninen, 1938.) Monostomate, with simple stylet, spined cuticula, four pairs of penetration glands, excretory vesicle V-shaped, flame-cell formula 2 [(1 + 1) + (1 + 1)], develop in oval to elongate sporocysts, penetrate the gills of *Callinectes sapidus* and migrate by hemal sinuses to encyst in various tissues; metacercariae develop with a flame-cell formula 2 [(2 + 2) + (2 + 2)]; adults experimentally in *Larus argentatus*, naturally in shore-birds. *(Cable and Hunninen, 1940.)

2. Cercaria of *Siphodera vinaledwardsi.* Monostomate, ocellate, pleurolophocercous, cuticula spined anterior to the ocelli, 7 pairs of penetration glands, long tail, bearing dorsal and ventral fin-folds, excretory system mesostomate with Y-shaped vesicle and flame-cell formula 2 [(2 + 2) + (2 + 2)], develop in saccate to elongate rediae; metacercariae encysted in flounders, especially *Paralichthys dentatus;* adults experimentally in toadfish, *Opsanus tau.* Species described by Linton, 1901 as *Monostomum vinal-edwardsi.* *(Cable and Hunninen, 1942a.)

3. Cercaria of *Deropristis inflata.* Distomate, ocellate, modified trichocercous, cuticula spinose anterior to the ocelli, excretory vesicle saccate, flame-cell formula 2 [(3 + 5 + 7) + (3 + 3 + 5)], develop in cylindrical, clavate to pyriform rediae; metacercariae encysted chiefly in the body region of *Nereis virens;* adults experimentally in eels, *Anguilla rostrata.* *(Cable and Hunninen, 1942b.)

In *CUMINGIA TELLINOIDES* and *TELLINA TENERA:*

1. Cercaria of *Monorcheides cumingiae.* Distomate, ocellate, spined cuticula, 2 pairs of penetration glands, tail with lateral lappets, excretory vesicle saccate and flame-cell formula 2 [(2 + 2) + (2 + 2)], develop in sporocysts, are expelled through the excurrent siphon; metacercariae encysted in the wall of the siphon and other tissues of *C. tellinoides* and *T. tenera;* adults in eels and flounders. *(Martin, 1938), life-cycle, Martin, 1940.

In *GEMMA GEMMA:*

1. Cercaria of *Parvatrema borealis.* Distomate, furcocercous, spined cuticula, oral sucker with small, lateral, ear-like projections, ceca short and wide, excretory vesicle V-shaped with arms extending to the pharyngeal level and filled with concretions, flame-cell formula 2 [2 + 2) + (2 + 2)], develop in oval to pyriform to cylindrical sporocysts with apical birth-pores; metacercariae unencysted on the mantle; adults in eider ducklings. *(Stunkard and Uzmann, 1958.)

2. *Cercaria adranocerca* Stunkard and Uzmann, 1959. Distomate, microcercous, spined cuticula, excretory vesicle oval, flame-cell formula 2 [(2 + 2) + (2 +2)], develop in sporocysts with apical birth-pores; life-cycle unknown. *(Stunkard and Uzman, 1959.)

In *HYDROBIA MINUTA:*

1. Cercaria of *Himasthla compacta.* Distomate, with reniform collar bearing 29 peristomial spines, 5 pairs of penetration glands, spined cuticula, tail simple with excretory pores on the sides, develop in simple rediae; metacercariae encysted

in *Mya arenaria* and other bivalves; adults experimentally in *L. argentatus.* *(Stunkard, 1960a.)

2. Cercaria of *Homalometron pallidum.* Distomate, ocellate, cuticula spined, 3 pairs of setae and dorso-ventral fin-folds on tail, stenostomate excretory system, saccate vesicle and flame-cell formula 2 [(3 + 3 + 3) + (3 + 3 +3)], develop in simple rediae; metacercariae encysted in *H. minuta, G. gemma,* and small polychaete annelids; adults in the intestine of *Fundulus* spp. and other fishes. *(Stunkard, 1964a.)

3. Cercaria of *Levinseniella minuta.* Monostomate, tailless, smooth cuticula, develop in oval to elongate sporocysts, leave the sporocyst in immature condition, do not emerge but encyst in snail-host; no second intermediate host; adults from scaup duck, *Nyroca affinis,* West Indies, and experimentally in white mice and hamsters. *(Stunkard, 1958.)

4. Cercaria of *Notocotylus minutus.* (Adult described as *N. gibbus* by Stunkard and Dunihue (1931) and as *N. imbricatus* by Harwood (1939).) Monostomate, ocellate, with small tail and ventrolateral locomotor pockets, excretory system of Yenchingensis type with anterior projection of the vesicle between the ocelli, develop in simple rediae; metacercariae encysted on shells and opercula of snail hosts and shells of *G. gemma,* no second intermediate host; adults in intestine of eider and domestic ducklings and in *L. argentatus.* *(Stunkard, 1960b.)

5. Cercaria of *Microphallus limuli.* Monostomate, with simple stylet and spined cuticula, 4 pairs of penetration glands, small tail, V-shaped excretory vesicle and flame cell formula 2 [(1 + 1) + (1 + 1)], metacercariae encysted in horseshoe crabs, *Limulus polyphemus;* adults in gulls, *L. argentatus,* experimentally in white mice. *(Stunkard, 1968.)

In *HYDROBIA SALSA:*

1. Cercaria of *Notocotylus atlanticus.* Monostomate, ocellate, small tail, ventrolateral locomotor pockets. Yenchingensis type excretory system with anterior projection of the vesicle, develop in simple rediae; metacercariae encysted on shells and opercula of hosts or other hard surfaces, no secondary intermediate host, adults in shore-birds and experimentally in eider and domestic ducklings. *(Stunkard, 1966b.)

2. Cercaria of *Notocotylus minutus.* (See *Hydrobia minuta.*)

3. Cercaria of *Paramonostomum alveatum.* Monostomate, ocellate, slender tail, locomotor-appendage pits on posterolateral ends of body, Monostomi type excretory system with simple ring vesicle; develop in simple rediae; metacercariae encysted on shells and opercula of host-snails and other hard surfaces; adults in eider and domestic ducklings. *(Stunkard, 1967a.)

4. Cercaria of *Paramonostomum parvum.* Monostomate, ocellate, small tail, accumulate on light side of container, simple ring-type excretory vesicle, ventrolateral locomotor pockets, develop in simple rediae; metacercariae encysted on shells and opercula of host-snails and other hard surfaces, no secondary intermediate host; adults in domestic and eider ducklings. *(Stunkard, 1967a.)

5. Cercaria of *Uniserialis breviserialis.* Monostomate, triocellate, small tail, ventrolateral locomotor pockets, excretory vesicle with an anterior loop around the median ocellus, develop in simple rediae; metacercariae encysted on shells and

opercula of host snails, do not grow, no secondary intermediate hosts, are infective immediately; mature in 2 weeks in domestic ducklings and day-old chicks. *(Stunkard, 1967b.)

In *HYDROIDES DIANTHUS:*

1. *Cercaria loossi* Stunkard, 1926. Monostomate, furcocercous, with dorsal fin-fold, develop in sporocysts in the coelom of *H. dianthus*, further development unknown. *(Martin, 1944.)

In *LAEVICARDIUM MORTONI:*

1. *Cercaria laevicardium* Martin, 1945. Distomate, trichocercous, cuticula unspined, V-shaped excretory vesicle and flame-cell formula 2 [(3 + 3) + (3 + 3)], develop in elongate sporocysts; unencysted metacercariae in ctenophores, *Mnemiopsis leidyi;* adults unknown. *(Martin, 1945.)

In *LITTORINA LITTOREA:*

1. Cercaria of *Cryptocotyle lingua*. Monostomate, ocellate, pleurolophocerous, develop in simple rediae, sigmoid excretory vesicle and flame-cell formula 2 [(3 + 7 + 7) + (7 + 7 + 7)], metacercariae encysted in the skin of fishes where they grow and develop an acetabulum; adults in birds, *L. argentatus* and *Sterna hirundo,* experimentally also in mammals, dogs and rats. *(Stunkard, 1930.)

2. *Cercaria parvicaudata* Stunkard and Shaw, 1931. Distomate, spined cuticula, small stylet, simple tail, 6 pairs of penetration glands, Y-shaped excretory vesicle with collecting ducts opening into the stem of the Y, flame-cell formula 2 [(3 + 3 + 3) + (3 + 3 + 3)], encyst in *Littorina* spp., *Nereis virens*, and *Euplana gracilis;* adults unknown. *(Stunkard, 1950.)

In *LITTORINA OBTUSATA:*

1. Cercaria of *C. lingua.*

2. Cercaria *parvicaudata.*

3. Cercaria of *Himasthla littorinae*. Distomate, reniform collar bearing 29 peristomial spines, excretory pores on sides of tail, no response to light, excretory vesicle saccate, collecting ducts with concretions, flame-cell formula 2 [(5 + 5 + 5 + 5 + 5) + (5 + 5 + 5 + 5 + 5)], develop in simple rediae; metacercariae encysted in palps, gills and mantle of *M. edulis, M. arenaria* and *Littorina* spp.; adults in 12 days in *L. argentatus*. *(Stunkard, 1966a.)

4. Cercaria of *Microphallus similis*. (Syn. *Cercaria ubiquita* Lebour, 1907, metacercaria in *Carcinus maenas* of M'Intosh, 1865, adult *Levinsenia pygmaeum similis* Jägerskiöld, 1900.) Monostomate, cuticula spined, simple stylet, small simple tail, 4 pairs of penetration glands, V-shaped excretory vesicle and flame-cell formula 2 [(1 + 1) + (1 + 1)], doubled in the metacercaria; metacercariae in crustaceans especially *Carcinus maenas*, adults in *L. argentatus* and *S. hirundo,* experimentally in white mice. *(Stunkard, 1957.)

In *LITTORINA SAXATILIS:*

1. Cercaria of *Podoctoyle atomon.* Distomate, cotylomicrocercous, with double pointed stylet, 3 pairs of penetration glands, smooth cuticula, excretory system mesostomate, vesicle saccate with flame-cell formula 2 [(2 + 2) + (2 + 2)], develop in elongate sporocysts; metacercariae encysted in amphipods, especially *Gammarus* spp.; adults experimentally in eels and sticklebacks, *Apeltes quadracus.* *(Hunninen and Cable, 1943a.)
2. Cercaria of *C. lingua.*
3. Cercaria of *M. similis.*
4. *Cercaria parvicaudata.*

In *MITRELLA LUNATA:*

1. Cercaria of *Opecoeloides manteri.* (Described as *Anisoporus manteri.*) Distomate, with double pointed stylet, smooth cuticula, modified cotylomicrocercous, mesostomate excretory system, saccate vesicle with flame-cell formula 2 [(2 + 2) + (2 + 2)], develop in elongate sporocysts; metacercariae in amphipods, *Carinogammarus mucronatus* and *Amphithoe longimana;* adults in *Fundulus* spp., *Apeltes quadracus, Paralichthys dentatus, Syngnathus fuscus* and *Hippoglossoides platessoides.* *(Hunninen and Cable, 1941.)
2. Cercaria of *Zoogonoides laevis.* Distomate, tailless, with simple stylet, smooth cuticula, excretory system mesostomate, vesicle saccate and flame-cell formula 2 [(2 + 2) + (2 + 2)]; metacercariae encysted in parapodia of *Nereis virens;* adults in *Tautoga onitis,* eggs without shells, hatch in sea-water and miracidia penetrate the snails. Two sporocyst generations. *(Stunkard, 1943.)

In *MYA ARENARIA:*

1. *Cercaria myae* Uzmann, 1952. Distomate, furcocercous, cuticula spined, short, wide ceca, 2 pairs of penetration glands, excretory vesicle V-shaped with long arms, pores at tips of the furcae, develop in motile, unpigmented, clavate sporocysts in the digestive gland and gonads of *Mya arenaria;* adult unknown. *(Uzmann, 1952.)

In *MYTILUS EDULIS:*

1. Cercaria of *Proctoeces maculatus.* (Syn. *Cercaria milfordensis* Uzmann, 1953.) Distomate, microcercous, cuticula smooth, 6 pairs of penetration glands, Y-shaped excretory vesicle with long arms and flame-cell formula 2 [(2 + 2) + (2+ 2)], develop in simple saccate, motile, orange-colored sporocysts; metacercariae become gravid in *M. edulis* and occur naturally in *Labrus merula* and *Calamus* spp., fishes which ingest mollusks. *(Uzmann, 1953), life cycle, Stunkard and Uzmann, 1959.

In *NASSARIUS OBSOLETUS:*

1. Cercaria of *Gynaecotyla nassicola.* [Syn. *Cercaria nassicola* Cable and Hunninen, 1938, *Cornucopula nassicola* (Cable and Hunninen) Rankin, 1939,

Gynaecotyla nassicola (Cable and Hunninen) Rankin, 1940.] Monostomate, with hastate stylet, cuticula spined, 4 pairs of penetration glands, develop in oval sporocysts; encysted metacercariae in the pericardial cavity of *Talorchestia longicornis;* adults in shore-birds, plovers, sandpipers, and experimentally in gulls. *(Rankin, 1940.)

2. Cercaria of *Himasthla quissetensis.* (*Cercaria quissetensis* Miller and Northup, 1926.) Distomate, with reniform collar bearing 31 peristomial spines, spined cuticula, simple tail with excretory pores on sides, develop in simple rediae; metacercariae in various mollusks; adults in gulls. *(Stunkard, 1938a.)

3. Cercaria of *Lepocreadium setiferoides.* (Syn. *Cercaria setiferoides* Miller and Northup, 1926.) Distomate, ocellate, trichocercous, cuticula spined, excretory vesicle tubular, contains concretions and extends anterior to the acetabulum; metacercariae in turbellarians and polychaete annelids; adults in flounders and sand-dabs. *(Martin, 1938.)

4. Cercaria of *Stephanostomum dentatum.* (Syn. *Cercaria dipterocerca* Miller and Northup, 1926.) Distomate, ocellate, spined cuticula, tail long with lateral and dorsal fins, 54 peristomial spines in two alternating rows, develop in rediae; metacercariae encysted in *Fundulus* spp. and *Menidia menidia;* adults in the intestine of *Paralichthys dentatus.* *(Stunkard, 1961.)

5. Cercaria of *Stephanostomum tenue.* Distomate, ocellate, spined cuticula, 42 peristomial spines in 2 alternating rows, hastate stylet, simple tail, develop in liver of *M. menidia;* adults in intestine of *Spheroides maculatus* and other fishes. *(Martin, 1939.)

6. Cercaria of *Microbilharzia variglandis.* (Syn. *Cercaria variglandis* Miller and Northup, 1926; to *Austrobilharzia* Johnston, 1917 by Penner, 1953.) Distomate, ocellate, furcocercous, schistosome, cuticula spined, 6 pairs penetration glands, develop in simple sporocysts; no metacercarial stage; adults dioecious in mesenteric veins of scaup ducks and experimentally in canaries, pigeons, ducks and gulls. *(Stunkard and Hinchliffe, 1952.)

7. Cercaria of *Zoögonus lasius.* (Syn. *Cercariaeum lintoni* Miller and Northup, 1926.) Distomate, tailless, spined, with simple stylet, attach to substrate by tip of tail and creep by attachment of alternate ends of the body; develop in simple sporocysts; metacercariae encysted in parapodia of *N. virens;* adults in *Anguilla rostrata, Opsanus tau* and other fishes. Eggs without shells, miracidia emerge in sea-water, penetrate snails and develop sporocyst generations. *(Stunkard, 1938b), 1941.

8. *Cercaria nassa* Martin, 1945. Distomate, furcocercous, spined, develop in sporocysts; life-cycle unknown. *(Martin, 1945.)

In *ODOSTOMIA TRIFIDIA:*

1. Cercaria of *Lecithaster confusus.* Cystophorous, hemiurid, enclosed in a discoid bulb with delicate filament, distomate, smooth cuticula, excretory vesicle Y-shaped with long arms, develop in elongate rediae; metacercariae not encysted in the haemocoele of *Acartia tonsa;* adults experimentally in *Fundulus* spp., and *Apeltes quadracus,* naturally in several other fishes. *(Hunninen and Cable, 1943b.)

In *AEQUIPECTEN IRRADIANS:*

1. An undescribed and unnamed furcocercous cercaria. *(Linton, 1915c.)

In *THAIS LAPILLUS:*

1. Cercaria of *Renicola thaidus.* Distomate, spined, tail small, simple, excretory vesicle Y-shaped, flame-cell formula $2 [(3 + 3 + 3 + (3 + 3 + 3)]$, develop in simple unbranched sporocysts, metacercariae encysted in *Mytilus edulis, Aequipecten irradians* and *Gemma gemma,* do not grow; adults experimentally in kidneys of *L. argentatus.* *(Stunkard, 1964b.)

In *UROSALPINX CINEREA:*

1. Cercaria of *Parorchis acanthus.* (*Cercaria sensifera* Stunkard and Shaw, 1931.) Described as *Parorchis avitus* Linton, 1914, who reported that the miracidia, still in the ova in the later folds of the uterus, contain each a single well-developed redia. Distomate, spined, with a peristomial collar, 6 pairs of penetration glands, excretory vesicle saccate, flame-cell formula $2 [(3 + 3 + 3) + (3 + 3 + 3)]$, pores on sides of the tail, develop in redia provided with locomotor-processes (feet); encyst in the open on any hard surface; adults in cloaca of *L. argentatus.* *(Stunkard and Shaw, 1931), life cycle, Stunkard and Cable, 1932.

LITERATURE CITED

Cable, R. M., and A. V. Hunninen, 1938. Observations on the life history of *Spelotrema nicolli,* n. sp. (Trematoda: Microphallidae) with the description of a new microphallid cercaria. *J. Parasitol.,* **24** (Suppl.): 29–30.

Cable, R. M., and A. V. Hunninen, 1940. Studies on the life history of *Spelotrema nicolli* (Trematoda: Microphallidae) with the description of a new microphallid cercaria. *Biol. Bull.,* **78**: 136–157.

Cable, R. M., and A. V. Hunninen, 1942a. Studies on the life history of *Siphodera vinaledwardsi* (Linton) (Trematoda: Cryptogonimidae). *J. Parasitol.,* **28**: 407–422.

Cable, R. M., and A. V. Hunninen, 1942b. Studies on *Deropristis inflata* (Molin), its life history and affinities to trematodes of the family Acanthocolpidae. *Biol. Bull.,* **82**: 292–312.

Harwood, P. D., 1939. Notes on Tennessee helminths, IV. North American trematodes of the subfamily Notocotylinae. *J. Tenn. Acad. Sci.,* **14**: 421–437.

Hunninen, A. V., and R. M. Cable, 1941. Studies on the life history of *Anisoporus manteri* Hunninen and Cable, 1940 (Trematoda: Allocreadiidae). *Biol. Bull.,* **80**: 415–428.

Hunninen, A. V., and R. M. Cable, 1943a. The life history of *Podocotyle atomon* (Rudolphi) (Trematoda: Opecoeliidae). *Trans. Amer. Microsc. Soc.,* **62**: 57–68.

Hunninen, A. V., and R. M. Cable, 1943b. The life history of *Lecithaster confusus* Odhner. (Trematoda: Hemiuridae). *J. Parasitol.,* **29**: 71–79.

Linton, E., 1900. Fish parasites collected at Woods Hole in 1898. *U. S. Fish. Comm. Bull.,* **1899**: 267–304.

Linton, E., 1901. Parasites of fishes of the Woods Hole region. *U. S. Fish. Comm. Bull.,* **1899**: 407–492.

Linton, E., 1914. Notes on a viviparous distome. *Proc. U. S. Nat. Mus.,* **46**: 551–555.

Linton, E., 1915a. *Tocotrema lingua* (Creplin), the adult stage of a skin parasite of the cunner and other fishes of the Woods Hole region. *J. Parasitol.,* **1**: 128–134.

Linton, E., 1915b. Sporocysts in an annelid. *Biol. Bull.,* **28**: 115–118.

Linton, E., 1915c. Notes on trematode sporocysts and cercariae in marine mollusks of the Woods Hole region. *Biol. Bull.,* **28**: 198–209.

Linton, E., 1940. Trematodes from fishes mainly from the Woods Hole region, Massachusetts. *Proc. U. S. Nat. Mus.* **88**: 1–172.

Martin, W. E., 1938. Studies on trematodes of Woods Hole; the life cycle of *Lepocreadium setiferoides* (Miller and Northup), Allocreadiidae, and the description of *Cercaria cumingiae*. *Biol. Bull.*, **75**: 463–474.

Martin, W. E., 1939. Studies on the trematodes of Woods Hole. II. The life cycle of *Stephanostomum tenue* (Linton). *Biol. Bull.*, **77**: 65–73.

Martin, W. E., 1940. Studies on the trematodes of Woods Hole. III. The life cycle of *Monorcheides cumingiae* (Martin) with special reference to its effect on the invertebrate host. *Biol. Bull.*, **79**: 131–144.

Martin, W. E., 1944. Studies on trematodes of Woods Hole. IV. Additional observations upon *Cercaria loossi* Stunkard developing in an annelid. *Trans. Amer. Microsc. Soc.*, **63**: 237–243.

Martin, W. E., 1945. Two new species of marine cercariae. *Trans. Amer. Microsc. Soc.*, **64**: 203–212.

Miller, H. M., and Flora E. Northup, 1926. The seasonal infestation of *Nassa obsoleta* (Say) with larval trematodes. *Biol. Bull.*, **50**: 490–509.

Penner, L. R., 1953. Experimental infections of avian hosts with *Cercaria littorinalinae* Penner, 1950. *J. Parasitol.*, **39** (Suppl.) : 20.

Rankin, J. S., Jr., 1940. Studies on the trematode family Microphallidae Travassos, 1921. III. The life cycle and ecology of *Gynaecotyla nassicola* (Cable and Hunninen) Yamaguti, 1939. *Biol. Bull.*, **97**: 439–451.

Ryder, J. A., 1884. On a skin parasite of the cunner (*Ctenolabrus adspersus*). *U. S. Fish. Comm. Bull.*, **1884**: 37–42.

Stafford, J., 1905. Trematodes from Canadian vertebrates. *Zool. Anz.*, **28**: 681–694.

Stunkard, H. W., 1929. Further observations on the cercaria which occurs in the marine annelid, *Hydroides dianthus*. *Anat. Rec.*, **44**: 264.

Stunkard, H. W., 1930. The life history of *Cryptocotyle lingua* (Creplin), with notes on the physiology of the metacercariae. *J. Morphol. Physiol.*, **50**: 143–191.

Stunkard, H. W., 1938a. The morphology and life cycle of *Himasthla quissetensis* (Miller and Northup, 1926). *Biol. Bull.*, **75**: 145–164.

Stunkard, H. W., 1938b. *Distomum lasium* Leidy, 1891 (syn. *Cercariaeum lintoni* Miller and Northup, 1926) the larval stage of *Zoögonus rubellus* (Olsson, 1868) (syn. *Z. mirus* Looss, 1901). *Biol. Bull.*, **75**: 308–334.

Stunkard, H. W., 1941. Specificity and host-relations in the trematode genus *Zoögonus*. *Biol. Bull.*, **81**: 205–214.

Stunkard, H. W., 1943. The morphology and life history of the digenetic trematode, *Zoögonoides laevis* Linton, 1940. *Biol. Bull.*, **85**: 227–237.

Stunkard, H. W., 1950. Further observations on *Cercaria parvicaudata* Stunkard and Shaw, 1931. *Biol. Bull.*, **99**: 136–142.

Stunkard, H. W., 1957. The morphology and life history of the digenetic trematode, *Microphallus similis* (Jägerskiöld, 1900) Baer, 1943. *Biol. Bull.*, **112**: 254–266.

Stunkard, H. W., 1958. The morphology and life history of *Levinseniella minuta* (Trematoda: Microphallidae). *J. Parasitol.*, **44**: 225–230.

Stunkard, H. W., 1960a. Further studies on the genus *Himasthla* with descriptions of *H. mcintoshi* n. sp., *H. piscicola* n. sp., and stages in the life-history of *H. compacta* n. sp. *Biol. Bull.*, **119**: 529–549.

Stunkard, H. W., 1960b. Studies on the morphology and life-history of *Notocotylus minutus* n. sp., a digenetic trematode from ducks. *J. Parasitol.*, **46**: 803–809.

Stunkard, H. W., 1960c. Thte morphology and life-history of the digenetic trematode, *Asymphylodora amnicolae* n. sp.; the possible significance of progenesis for the phylogeny of the Digenea. *Biol. Bull.*, **117**: 562–581.

Stunkard, H. W., 1961. *Cercaria dipterocerca* Miller and Northup, 1926 and *Stephanostomum dentatum* (Linton, 1900) Manter, 1931. *Biol. Bull.*, **120**: 221–237.

Stunkard, H. W., 1964a. The morphology, life-history, and systematics of the digenetic trematode, *Homalometron pallidum* Stafford, 1904. *Biol. Bull.*, **126**: 163–173.

Stunkard, H. W., 1964b. Studies on the genus *Renicola:* observations on the life-history, specificity, and systematic position. *Biol. Bull.*, **126**: 467–489.

STUNKARD, H. W., 1966a. The morphology and life-history of *Himasthla littorinae* n. sp. (Echinostomatidae). *J. Parasitol.,* **25**: 367–372.

STUNKARD, H. W., 1966b. The morphology and life-history of *Notocotylus atlanticus* n. sp., a digenetic trematode of eider ducks, *Somateria mollissima*, and the designation, *Notocotylus duboisi nom nov.*, for *Notocotylus imbricatus* (Looss, 1893) Szidat, 1935. *Biol. Bull.,* **131**: 501–515.

STUNKARD, H. W., 1967a. Studies on the trematode genus *Paramonostomum* Lühe, 1909 (Notocotylidae). *Biol. Bull.,* **132**: 133–145.

STUNKARD, H. W., 1967b. The morphology, life-history, and systematic relations of the digenetic trematode, *Uniserialis breviserialis sp. nov.,* (Notocotylidae) a parasite of the bursa Fabricius of birds. *Biol. Bull.,* **132**: 266–276.

STUNKARD, H. W., 1968. The asexual generations, life-cycle, and systematic relations of *Microphallus limuli* Stunkard, 1951 (Trematoda: Digenea). *Biol. Bull.,* **134**: 332–343.

STUNKARD, H. W., 1969. The morphology and life-history of *Neopechona pyriforme* (Linton, 1900) n. gen., n. comb., (Trematoda: Lepocreadiidae). *Biol. Bull.,* **136**: 96–113.

STUNKARD, H. W., AND R. M. CABLE, 1932. The life history of *Parorchis avitus* Linton, a trematode from the cloaca of the gull. *Biol. Bull.,* **62**: 328–338.

STUNKARD, H. W., AND F. W. DUNIHUE, 1931. Notes on trematodes from a Long Island duck with description of a new species. *Biol. Bull.,* **60**: 179–186.

STUNKARD, H. W., AND M. C. HINCHLIFFE, 1952. The morphology and life history of *Microphallus variglandis* (Miller and Northup, 1926) Stunkard and Hinchcliffe, 1951, avian blood-flukes whose larvae cause "swimmer's itch" of ocean beaches. *J. Parasitol.,* **38**: 248–265.

STUNKARD, H. W., AND C. RUTH SHAW, 1931. The effect of dilution of sea-water on the activity and longevity of certain marine cercariae, with descriptions of two new species. *Biol. Bull.,* **61**: 242–271.

STUNKARD, H. W., AND J. R. UZMANN, 1958. Studies on digenetic trematodes of the genera *Gymnophallus* and *Parvatrema. Biol. Bull.,* **115**: 276–302.

STUNKARD, H. W., AND J. R. UZMANN, 1959. The life cycle of the digenetic trematode, *Proctoeces maculatus* (Looss, 1901) Odhner, 1911 [syn. *P. subtenuis* (Linton, 1907) Hanson, 1950], and a description of *Cercaria adranocerca* n. sp. *Biol. Bull.,* **116**: 184–193.

UZMANN, J. R., 1952. *Cercaria myae sp. nov.*, a fork-tailed larva from the marine bivalve, *Mya arenaria. J. Parasitol.,* **38**: 161–164.

UZMANN, J. R., 1953. *Cercaria milfordensis nov. sp.*, a microcercous trematode larva from a marine bivalve, *Mytilus edulis* L., with special reference to its effect on the host. *J. Parasitol.,* **39**: 445–451.

VERRILL, A. E., AND S. I. SMITH, 1873. Report upon the invertebrate animals of Vineyard Sound and adjacent waters. *Rep. U. S. Comm. Fish.,* **1871**: 295–778.

Reference: *Biol. Bull.*, **138**: 77-87. (February, 1970)

THE ROLE OF ILLUMINATION AND TEMPERATURE IN THE CONTROL OF SEXUAL REPRODUCTION IN THE PLANARIAN *DUGESIA TIGRINA* (GIRARD)

C. VOWINCKEL

Department of Zoology, McGill University, Montreal, Canada

It is well established that the timing of reproductive activity in higher animals such as vertebrates and arthropods is dependent on environmental stimuli which set in train various physiological mechanisms. In lower organisms little exact knowledge is so far available on the nature of such controlling stimuli.

Planarians belong among the most primitive Bilateria. In spite of their apparent simplicity they have envolved morphologically complex organs of reproduction and, in many species, a seasonal alternation of reproduction between sexual and asexual pathways.

Such a species is *Dugesia tigrina* (Girard). In the St. Lawrence River it develops its reproductive organs in fall, lays cocoons in early summer and reproduces asexually by fission for the rest of the warm season. The object of the present enquiry is the nature of the stimulus that will induce the change from asexual reproduction to the development of sex organs in late summer in this species.

Previous experimentation on the action of environmental agents on the induction of sexual development by Castle (1928), and Hyman (1934ab) emphasized the importance, for planarians, of low water temperatures, which appears well established. Vowinckel (1970) showed that exposure to such temperatures stimulates an increase in germ cells associated in testicular primordia and that this activity is transmittable via homogenates.

In view, however, of the widespread control exerted by day length over the seasonal reproductive cycle of many animal groups it appears conceivable that sexual reproduction in planarians is regulated not only by temperature but controlled additionally by photoperiod. The work reported below puts this concept to a test.

MATERIALS AND METHODS

Dugesia tigrina was collected from its natural habitat in late summer and maintained at 27° C till November. During this time sexual structures already acquired under natural conditions disappeared slowly and the population finally returned to asexual reproduction. Fission products were not removed and when experimentation began the population consisted partly of individuals grown up under natural habitat conditions and partly of individuals reared under incubator conditions. This population was used for experiment 1 (series A-D).

The population was maintained in white enamel pans in a 9 cubic foot incubator at 27° C and a constant photoperiod of LD 16:8 (ON from 0600 to 2200). Nine inch fluorescent tubes served as light sources. They were controlled by automatic clock switches and were reset daily in series which experienced photoperiod variations. In the first experiment discussed below, series A and C (Figure I, Table I), light conditions were varied as follows: from 16 hours daylight the photoperiod was curtailed by 7–8 minutes every morning and evening so that each successive day was 15 minutes shorter. In addition the light intensity was reduced in 3 steps from 1500 lux to 750, 280, and 175 lux approximately by enclosing the fluorescent light with sheets of paper. When the photoperiod had reached LD 8:16 the whole process was reversed until LD 16:8 and full intensity were reestablished.

In the second experiment, series E (Table II), day length decrease followed the same regime, light intensity was decreased in 9 small steps from 1500 lux to 200 lux approximately, using a shutter which gradually closed over the fluorescent light.

The temperature was decreased by approximately 1° C every second day in the first experiment. This took the planarians from 27° C to 12° C in 30 days. The temperature regime for experiment 2 is given in Table II.

One individual from this population, after laying cocoons in the laboratory, was used to rear a clone from its fission products. This clone was maintained also at LD 16:8 but at 24° C since this temperature more closely corresponds to summer conditions in the population's natural habitat. This clone supplied the material for the second experiment (series E-G).

All animals were fed veal liver 3 times a week and their culture water replaced from bottles kept within the incubator. Unchlorinated river water was used throughout.

The experiments reported below were conducted in four 4 cubic foot incubators, series A, B, C and D concurrently and series E, F and G later. Experimental animals were maintained in 9 × 5 cm glass petri dishes with black lids.

Evaluation methods: The degree of sexual development was determined histologically. Animals were fixed in Bouin's fixative, wax-embedded under vacuum, serial sections cut a 16 micra and stained with aldehyde fuchsin using phloxin as counter stain.

A scale of 10 stages was developed for evaluation by Vowinckel (1968) and is given below in abridged form:

stage 1:	2–5 cells typical size of testicular primordia
stage 2:	6–10 cells typical size of testicular primordia
stage 3:	11–20 cells typical size of testicular primordia
stage 4:	maturation divisions begin in most testicular follicles, testicular lumen forms
stage 5:	most testicular follicles contain mature sperm
stage 6:	future copulatory complex indicated by a round cavity
stage 7:	penis mound recognizable within this cavity
stage 8:	female antrum has arisen posterior to male antrum
stage 9:	bursal stalk and bursa have appeared, gonopore opens
stage 10:	ovovitelline duct and sperm duct have connected with copulatory complex

RESULTS

Four groups of 45 planarians each were randomly selected from the population collected in the natural habitat. All showed signs of recent fission. They were exposed to 4 different temperature and light regimens. In one group both factors were varied, in two groups only one factor was varied and in the last group both factors were held constant. The variations all followed the same scheme: the factor was reduced for 30 days at a constant daily rate and then the trend was reversed and the factor increased again at the same rate.

When temperature or light conditions were not varied they were held at 27° C and LD 16:8, the animals therefore experienced no change in these parameters from the conditions in the stock incubator.

In the four series the combinations of light and temperature regimens were:

A: light varied, temperature varied.
B: light varied, temperature not varied.
C: light not varied, temperature varied.
D: light not varied, temperature not varied.

Animals were sacrificed at increasing intervals: twice daily (first 3 days), daily (next 3 days) every second day (for 38 days) every third day (for 24 days) and all remaining individuals on day 72. Serial sections were then scanned for the degree of sexual development.

The results of this evaluation are given in Figure 1. Sexual development resulted when either or both factors were varied (series A, B, C), no sexual development resulted if both factors were held constant (series D). There are some differences between those series that responded positively. When both light and temperature were varied (series A) 9 individuals reached marked testicular development (stage 3 or more). When only light was varied 6 individuals reached stage 3 or more and if temperature alone was varied 11 individuals reached or surpassed stage 3.

In order to evaluate further the differences between the series a count was made of all sections of testicular follicles containing spermatids and/or mature sperm in each worm. This count is given in Table I. It shows clearly that in series A (both factors varied) no individual reached testicular maturation. In contrast photoperiod variations alone (series B) caused fewer individuals to respond but testis maturation was reached, at least in some follicles, in all animals that responded.

During the experiment all fission products were counted and removed. The count was checked against the number of worms which had fissioned. The results are given in Figure 2 where the count is expressed as per cent of the existing population and summed over successive periods of 5 days. The fission rate demonstrates the basic difference between the animals which only received illumination variations (B) and those which received temperature variations (A and C). The series which received changes in daylight only continued to fission throughout the duration of the experiment as did the control series D. Both series that were exposed to decreases in temperature on the other hand ceased to fission until conditions again approached those of the control series.

Since series B (illumination variations only) continues to fission no stage higher than stage 5 can be reached by this group. The developing copulatory complex is always lost with the fission product. Normally testicular development continues after stage 5 by growth of follicles and by maturation of late developing

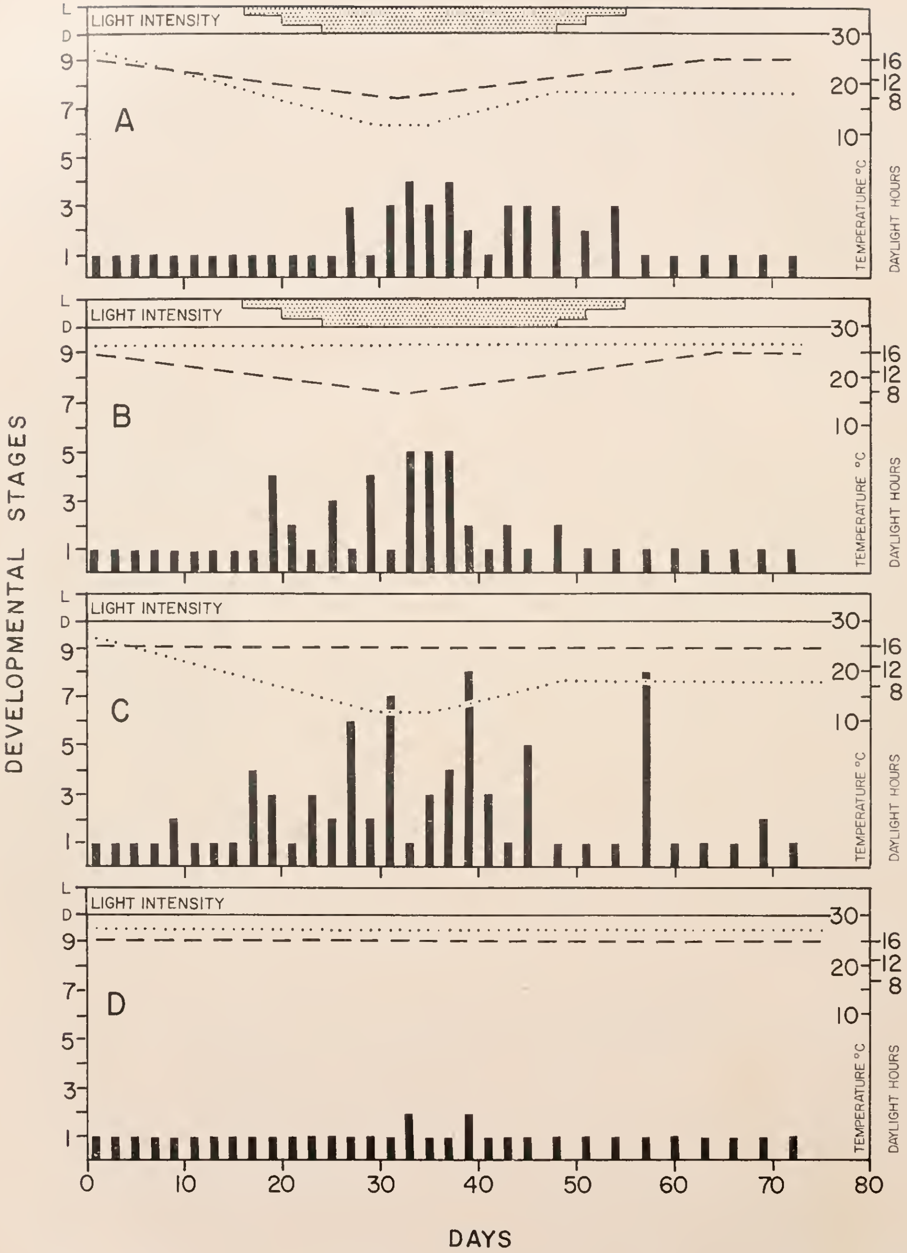


Figure 1. Degree of sexual development of the natural population when daylight and/or temperature are progressively decreased. Each column represents one individual; - - - - photoperiod, ... temperature. Not all individuals on days 1–6 and after day 69 are entered. Individuals that were omitted all reached stage 1 only.

TABLE I

Number of cross sections of testis follicles per worm containing spermatids and/or mature sperm. Total number of worms containing sperm in series A = 0, B = 7, C = 6, D = 0. Population collected from natural habitat

Series	A	B	C	D	Number of animals/series
	Light varied temp. varied	Light varied temp. const.	Light const. temp. varied	Light const. temp. const.	
Day					
1–15	0	0	0	0	14
17	0	0	95	0	1
19	0	2	0	0	1
21	0	0	0	0	1
23	0	0	0	0	1
25	0	25	0	0	1
27	0	0	91	0	1
29	0	3	0	0	1
31	0	5	592	0	1
33	0	402	0	0	1
35	0	108	0	0	1
37	0	364	0	0	1
39	0	0	84	0	1
41	0	0	0	0	1
43	0	0	0	0	1
45	0	0	37	0	1
48	0	0	0	0	1
51	0	0	0	0	1
54	0	0	0	0	1
57	0	0	804	0	1
60–72	0	0	0	0	9–12
	0	909	1703	0	42–45

follicles. For this reason the number of follicular sections containing sperm which are typical for stages 6–8 in series C (temperature variations only) are in series B found with stage 5, as an abridged rearrangement of Table I shows:

	Stage	Sections c sperm	Day		Stage	Sections c sperm	Day
Series B	5	402	33	Series C	5	37	45
(light only	5	108	35	(temperature	6	91	27
varied)	5	364	37	only varied)	7	541	31
					8	84	39
					8	804	57

Three series of this experiment (B, C, D) were repeated with incubator reared, cloned animals which descended from an individual which had previously laid viable cocoons in the laboratory. These 3 series were conducted as follows:

Series E: light conditions varied, temperature maintained constant.
Series F: light conditions maintained constant at full intensity; temperature decreased.
Series G: light conditions maintained constant at full intensity; temperature maintained constant at 24° C.

TABLE II

Degree of sexual development of incubator reared individuals exposed to gradually reduced illumination and temperature conditions. Stage = degree of development reached by individual fixed on date indicated. For explanation of stages see text. Series G: neither temperature nor light varied, series E: light varied only, series F: temperature varied only

Day	Series G				E					F			
	Number of fission products	Stage	Temperature (Centigrade)	Daylength (hr min)	Number of fission products	Stage	Light intensity reduced	Temperature (Centigrade)	Daylength (hr min)	Number of fission products	Stage	Temperature (Centigrade)	Daylength (hr min)
1			23.2	16.00				23.5	16.00			23.5	16.00
2	2		22.8	16.00	13			23.5	15.52	0		23.0	16.00
3	5		23.3	16.00	2		x	23.7	15.37	0	2	23.1	16.00
4	3		23.3	16.00	6			23.7	15.22	7		24.1	16.00
5	3		23.3	16.00	1	2		23.6	15.07	1		24.5	16.00
6	9		23.4	16.00	0			23.5	14.52	4		23.1	16.00
7	1		23.4	16.00	4		x	23.2	14.37	0	2	22.6	16.00
8	4	3	23.3	16.00	3			23.2	14.22	0		22.6	16.00
9	0		23.2	16.00	3	3		23.3	14.07	3		22.0	16.00
10	2		23.3	16.00	5			23.5	13.52	1		21.6	16.00
11	1		23.9	16.00	4		x	23.5	13.37	1	2	21.4	16.00
12	1	2	23.9	16.00	4			23.3	13.22	1		21.0	16.00
13	4		23.4	16.00	0	2		22.9	13.07	2		20.2	16.00
14	4		23.5	16.00	2			23.6	12.52	0		20.2	16.00
15	6		23.5	16.00	4		x	23.1	12.37	0	2	19.7	16.00
16	5	2	23.7	16.00	2			23.5	12.22	1		19.7	16.00
17	3		23.6	16.00	3	2		23.0	12.07	0		19.1	16.00
18	1		23.3	16.00	4			23.7	11.52	0		19.2	16.00
19	1		23.5	16.00	1		x	23.6	11.37	0	2	18.2	16.00
20	3	2	23.3	16.00	1			22.9	11.22	0		18.2	16.00

Table II—(*Continued*)

Day	Series G				E					F			
	Number of fission products	Stage	Temperature (Centigrade)	Daylength (hr min)	Number of fission products	Stage	Light intensity reduced	Temperature (Centigrade)	Daylength (hr min)	Number of fission products	Stage	Temperature (Centigrade)	Daylength (hr min)
21	2		23.6	16.00	3	2		23.3	11.07	0		17.6	16.00
22	5		23.5	16.00	5			23.2	10.52	0		17.7	16.00
23	2		23.5	16.00	1		x	23.3	10.37	0	2	17.3	16.00
24	3	2	23.3	16.00	1			23.7	10.22	0		17.3	16.00
25	3		23.4	16.00	1	2		23.7	10.07	0		17.0	16.00
26	1		23.5	16.00	4			23.8	9.52	0		16.8	16.00
27	4		23.3	16.00	3		x	22.8	9.37	0	3	16.3	16.00
28	3	2	23.5	16.00	7			23.5	9.22	0		16.2	16.00
29	9		23.4	16.00	1	2		23.8	9.07	0		15.6	16.00
30	0		23.4	16.00	1			24.1	8.52	0		15.8	16.00
31	3		23.3	16.00	2		x	22.8	8.37	0	3	14.8	16.00
32	1	2	23.6	16.00	0			23.3	8.22	0		15.0	16.00
33	0		23.5	16.00	3	2		23.2	8.07	0		14.0	16.00
34					2			23.1	7.52	0		14.1	16.00
35					1		x	23.3	7.37	0	3	14.1	16.00
36					8			22.8	7.22	0		14.3	16.00
37					1	2		22.9	7.07	0		13.8	16.00
38								23.3		0		13.8	16.00
39								23.3		0	3	14.0	16.00
40								23.2		0		13.2	16.00
41								23.2		0		13.1	16.00
42										0		12.8	16.00
43										0	2	12.8	16.00

Animals were sacrificed every fourth day and evaluation was done by the same method as before.

The results are given in Table II and show plainly that in spite of the fact that conditions are closely similar to series B-D no sexual development resulted beyond stage 3 in the cold exposed series (F) and that the changes in photoperiod resulted not even in a size increase of testicular primordia. The fission rate responds in a normal manner. In series F fission ceases as the temperature drops below 20° C and persists in series E and G where the temperature remains at 24° C.

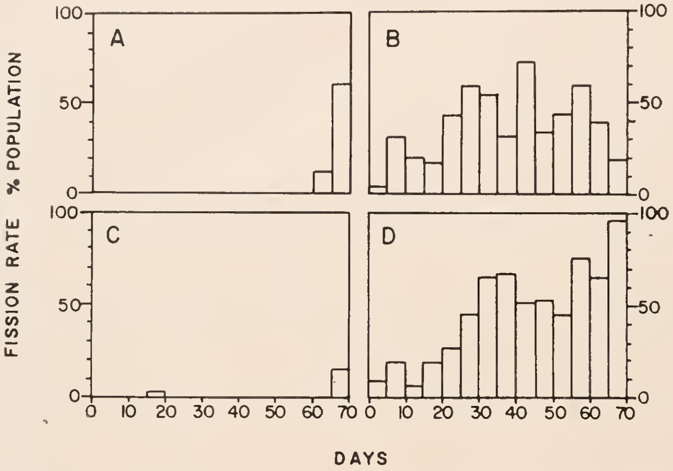


FIGURE 2. Fission rates in the four series of experiment 1 (natural population) expressed as per cent of population and summed over 5 day periods; series A: light and temperature varied, series B; light only varied, series C: temperature only varied, series D: neither light nor temperature varied.

Numerous further attempts with various temperature regimen, *e.g.*, fast drops in temperature, diurnal temperature variations with the daily mean temperature decreasing slowly and a succession of small stepwise decreases have all been equally unsuccessful in inducing sexual development in the incubator reared material.

DISCUSSION

The results of our experimentation indicate that changes in photoperiod as well as temperature can induce sexual development. The sensitivity of planarians to light is well established. Recently May and Birukow (1966) found that *D. gonocephala* has a variable preference for light intensities if given a choice. This preference varies in correlation with the phases of the moon during the synodial month. Ude (1964) correlated natural and reversed day and night rhythms with neurosecretory activity in *Dendrocoelum lacteum* and found that animals fixed during the light period differed from those fixed during the dark period by the number of cells showing histological signs of neurosecretory activity. Both Ude (1964) and Lender (1964) found that neurosecretory activity—as judged from staining affinities of nerve cells—is at a peak during the time when sex organs are developing in planarians. Our finding that a reduction in period and intensity of illumination can artificially induce sexual development therefore agrees well with their results.

The reason why in series A the combination of the two factors acts to retard sexual development (Table I) as compared to series B and C is not immediately obvious. A comparison was made with the natural habitat conditions (Vowinckel 1968). In 1967 daily temperature maxima were recorded at the place of collection of the population. On August 27 some members of the natural population already contained mature sperm in their testes. The highest water temperature of the summer, 25° C, was recorded on August 26 and the minimum on the night before for the first time fell below 20° C. The water temperatures at the time of induction of sexual development, therefore, are those of high summer. Daily illumination, on the other hand, at this time has been steadily reduced for two months, both in intensity and photoperiod. It appears therefore possible that under natural conditions changes in illumination represent the inducing stimulus while a decrease in temperature stimulates sexual development later in the season and inhibits fission.

It is known that temperature and photoperiod, within limits, interact, and can to some extent replace each other, as releasing stimuli in the induction of sexual development of organisms. Recent examples are given by Minis and Pittendrigh (1968) and Lees (1959, 1963).

In series B fission continues although differentiation towards sexual reproduction has proceeded to a point where all testis follicles contain mature sperm. This seems to indicate that two different mechanisms are involved in the control of asexual and sexual reproduction. If only one factor ruled both phenomena, acting as stimulant for one pathway and as inhibitor for the other, such overlap should not occur.

Brown (1965) and Brown and Park (1965) have brought evidence that changes in the horizontal magnetic field can be correlated with changes in the behavior of *D. dorotocephala*. They suggest that the regular cyclic variations of this field may be responsible for the timing of physiological rhythms. Unfortunately, under normal experimental conditions geomagnetic variations are not easily controlled. Using a gauss meter (Bell, model 620) we found that the earth's field was reduced to approximately 0.06 *g* inside the cabinet of our incubators. Since all four incubators were identical in make and were run concurrently on the same table it should be expected that any variations of the earth''s field had the same effect in all incubators. No change in the magnetic vector could be detected when the fluorescent lights were switched on or off. The sexual development resulting in series B should, therefore, be correlated with the changes in illumination. The cooling motors, on the other hand, gave a brief surge of 0.01 *g* whenever they were activated or deactivated. Series A and C, therefore, differed from series B and D not only in temperature but also in the properties of the magnetic vector since in the latter two series the cooling motors did not activate, 27° C being above room temperature. However, it is felt that in view of the well established influence of water temperature on planarian sexual development it would be precipitous to assign an inductive role to a factor which must have shown variations very different from the pattern of the natural environment.

Kenk (1940) suggested that planarians have an inherent rhythm of sexual reproduction, independent of environmental control. This suggestion was accepted by Goldsmith (1942) and Dahm (1958) but rejected by Hyman (1943b) who argued that it is possible to induce sexuality arhythmically by using temperature treatments. Our incubator reared descendents of a parent which had proven its ability to lay viable cocoons have never reproduced sexually although the clone is maintained now for over 3 years. From this evidence we deduce that sexual reproduction in *D. tigrina* cannot be due to an inherent rhythm but rather depends on environmental stimuli which under these special maintenance conditions are absent.

When mature, our incubator reared individuals neither respond to reductions in photoperiod nor to reductions in temperature closely similar to those eliciting positive results in individuals that grew up under natural conditions. The altered maintenance temperature could hardly be the cause for this difference in response since it resembles more closely the natural conditions. Our incubator reared animals appear healthy in every respect, have a normal fission rate (approximately once every 8 days) and respond to a drop in temperature with an increase in the number of germ cells associated in testicular primordia (Vowinckel, 1970) which suggests that they have not lost their ability to respond. Their presence in small numbers in the mixed population may explain why some individuals in series A-C fail to respond to the stimuli.

Since mature animals collected from the natural habitat in late summer regularly react to illumination or temperature reductions with sexual development (Hyman 1943a, Vowinckel 1968) an occurrence prior to collection must have "triggered" them to respond to later stimulation. The "setting of the trigger" may be possible only during a short period in the development of the planarian. At present we are investigating this possibility.

This work was supported by a McConnell Fellowship of McGill University to the author and a National Research Council of Canada grant to Professor Joan Marsden in whose laboratory the experiments were performed and to whom the author is indebted for valuable advice and criticism. Part of the work reported here was included in a Ph.D. thesis submitted to McGill University.

SUMMARY

1. Planarians were collected from the natural habitat in late summer and exposed to gradual reductions in illumination and/or temperature.

2. They responded to variations of either or both factors with sexual development while the control group under constant temperature and light conditions did not enter sexual development.

3. Asexual reproduction (fission) was inhibited by low temperatures and apparently not influenced by changes in illumination.

4. Incubator reared, cloned descendents of an individual which had previously laid viable cocoons did not respond to the same treatment.

5. The results are discussed and the importance of the difference between the two populations' response is interpreted.

LITERATURE CITED

Brown, F. A., Jr., 1965a. Duration of an after-effect in planarians following a reversed horizontal magnetic field. *Biol. Bull.,* **128**: 347–355.

Brown, F. A., Jr., and Y. H. Park, 1965b. Phase shifting a lunar rhythm in planarians by altering the horizontal magnetic vector. *Biol. Bull.,* **129**: 79–86.

Castle, W. A., 1928. An experimental and histological study of the life cycle of *Planaria velata. J. Exp. Zool.,* **51**: 417–483.

Dahm, A. G., 1958. *Taxonomy and Ecology of Five Species Groups in the Family Planariidae (Turbellaria, Tricladida, Paludicola).* Nya Lintografen 12158, Malmø, 241 pp.

Goldsmith, E. D., 1942. Sexuality in *Dugesia tigrina. Nature,* **150**: 351.

Hyman, L. H., 1943a. Environmental control of sexual reproduction in flatworms. *Anat. Rec.,* **81**: 108.

Hyman, L. H., 1943b. Water content of medusae, sexuality in a planarian. *Nature,* **151**: 140.

Kenk, R., 1940. The reproduction of *Dugesia tigrina* (Girard). *Amer. Natur.,* **74**: 471–475.

Lees, A. D., 1959. The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton—I The influence of these factors on apterous virginoparae and their progeny. *J. Insect Physiol.,* **3**: 92–117.

Lees, A. D., 1963. The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton—III Further properties of the maternal switching mechanism in the apterous aphids. *J. Insect Physiol.,* **9**: 153–164.

Lender, T., 1964. Mise en évidence et rôles de la neurosécrétion chez les planaires d'eau douce (Tourbellariés, Triclades). *Ann. Endocrinol.,* **25**(5): 61–65.

May, E., and G. Birukow, 1966. Lunarperiodische Schwankungen des Lichtpraeferendums bei der Bachplanarie. *Naturwissenschaften,* **53**: 182.

Minis, D. H., and C. S. Pittendrigh, 1968. Circadian oscillation controlling hatching: Its ontogeny during embryogenesis of a moth. *Science,* **159**: 534–536.

Ude, J., 1964. Untersuchungen zur Neurosekretion bei *Dendrocoelum lacteum,* Oerst. (Platyhelm. Turbell.). *Z. Wiss. Zool.,* **170**: 223–255.

Vowinckel, C., 1968. The induction of sexual development in the planarian *Dugesia tigrina* (Girard), *Doctoral dissertation, McGill University,* Montreal, 104 pp.

Vowinckel, C., 1970. The stimulation of germ cell proliferation in *Dugesia tigrina* (Girard), *J. Embryol. Exp. Morphol.,* in press.

Reference: *Biol. Bull.*, 138: 88–95. (February, 1970)

ON SEXUAL AGGLUTINATION AND MATING-TYPE SUBSTANCES (GAMONES) IN ISOGAMOUS HETEROTHALLIC CHLAMYDOMONADS. II. THE EFFECT OF CONCANAVALIN A UPON THE MATING-TYPE REACTION[1]

LUTZ WIESE AND DAVID W. SHOEMAKER[2]

Department of Biological Science, Florida State University, Tallahassee, Florida 32306

In the copulation of isogametes in *Chlamydomonas*, the initial contact between sexually different cells at fertilization occurs spatially localized in the mating-type reaction, a species-specific agglutination of the flagella tips. The responsible surface components, the mating-type substances, have been purified and act, in their isolated state, as isoagglutinins: the male component causes homosexual agglutination between gynogametes and the female component isoagglutinates androgametes (*cf.* Wiese, 1965, 1969). In these isoagglutinations, the initial contact between sexually different gametes is isolated in copy form and can be studied in the interaction between the isoagglutinins and the reacting gametes. Pairs of mating-type substances have been isolated from two sexually compatible and four sexually isolated taxons (*C. eugametos* and *C. moewusii* syngen I; *C. moewusii* syngen II, *C. reinhardti, C. mexicana,* and *C. chlamydogama*). Both isoagglutinins of a species are sex- and species-specific glycoproteins of complex nature. The physiological and chemical analysis of the isoagglutinins concentrates on their functional structure, particularly on the nature of their combining sites, on their mode of interaction, and on those features which condition the specificity of the agglutination mechanism as to sex and species.

The phytohemagglutinin Concanavalin A, a globulin of the jack bean *Canavalia ensiformis,* combines specifically with non-reducing terminal sugar residues of branched macromolecules (*cf.* Sumner and Howell, 1936; Goldstein, Hollerman and Merrick, 1965; Goldstein and So, 1965; So and Goldstein, 1968). Concanavalin A (CONA) seems to react exclusively with α-D-glucopyranosyl, β-D-fructofuranosyl, and α-D-mannopyranosyl residues. Unmodified hydroxyl groups on C_3, C_4 and C_6 are considered to be essential for establishing the contact with the combining site of the CONA-molecule (Goldstein, Hollerman and Smith, 1965; Goldstein and Iyer, 1966; So and Goldstein, 1967). The interaction is known to depend on bivalent cations (Agrawal and Goldstein, 1968). Analogous to an antibody, the binding capacity of CONA is inherent to two combining sites (So and Goldstein, 1968). Hence, CONA is able to form complexes with and to cause precipitation of polysaccharides, mucopolysaccharides, lipopolysaccharides and glycoproteins possessing those sugars in a terminal position. This substrate specificity of its binding action makes CONA an analytical tool for the characterization of functional components of carbohydrate nature. In addition, however, CONA may combine in an unspecified manner with various polyelectrolytes and with certain neutral polysaccharides which do not possess the terminal sugars mentioned (*cf.* Doyle, Woodside and Fishel, 1968). CONA was checked for its potency to react with the glycoproteinaceous components of the sexual agglutination mechanism in *Chlamydomonas*, the mating-type substances, and for its ability to provide information on the character of their combining sites.

---

[1] The financial support of this research by the National Science Foundation Grant No. GB 4921 is gratefully acknowledged.

[2] This work forms part of a dissertation to be submitted by David W. Shoemaker to Florida State University in partial fulfillment of the degree of Doctor of Philosophy.

MATERIAL AND METHODS

The effects of CONA on the mating-type reaction have been investigated in a test system with two sexually compatible taxons [*C. eugametos* (Indiana Collection Nos. 9 and 10, *cf.* Starr, 1964) and *C. moewusii* syngen I (Indiana Coll. Nos. 96 and 97)] and a third form, *C. moewusii* syngen II (Indiana Coll. Nos. 792 and 793), which is sexually isolated from the first two pairs of mating types. On the homologies between the mating types see below.

The cultivation of the organisms, the sexualization of the vegetative cultures, *i.e.*, the induction of gametogenesis, the preparation and purification of the isoagglutinins, and the test techniques have been described earlier (*cf.* Wiese, 1965).

The CONA preparation used was the commercially available product from Calbiochem (Los Angeles). CONA was applied in solutions of 0.02 *M* TRIS-buffer pH 7.2 or in 0.002 *M* phosphate buffer pH 6.7. Trypsin (salt-free, twice crystallized) (Worthington Enzymes, Freehold, New Jersey) was applied in 0.05 *M* TRIS-buffer at 26° C for 45 min at pH 7.76. The trypsin-inhibitor (Soy bean inhibitor, 5 × crystallized) was obtained from Nutritional Biochemical Corporation (Cleveland, Ohio). Experimental details are given in the text.

RESULTS

When gametes of *C. eugametos*, *C. moewusii* syngen I and *C. moewusii* syngen II are brought into a solution of 0.01% CONA, an instantaneous isoagglutination within each of the six gamete types occurs. In their typical form, the clusters of agglutinated gametes cannot be distinguished from normal sexual clumping between andro- and gynogametes, from the two isoagglutinations induced by the isolated mating-type substances (isoagglutinins), and from those isoagglutinations (Baker and Wiese, 1968) which are caused by antibodies against isolated mating-type substances. In addition to true isoagglutinations from flagella tip to flagella tip, there may occur a cluster formation in which the participating gametes adhere by means of their flagella tips to microscopically visible particles which have adsorbed CONA as, for instance, detached flagella. Such a reaction can be induced by coating sephadex beads with CONA molecules to which the gametes of all six sexes adhere. Uncoated sephadex does not adsorb any of the six gamete types. The ability of cells to combine with CONA can hence easily be judged in a system with CONA-coated sephadex beads.

The causation of clustering by isoagglutination and the unilateral inhibition of one mating-type activity, described below, are independent of another CONA-action on the cell's motility causing immobilization after 5–45 minutes, depending on the concentration of CONA and varying with the physiological state of the cells. The immobilization also ensues in both sexes of all three taxons.

When gametes of the three taxons are incubated with lower concentrations of CONA (0.001%), they no longer enter isoagglutination except in a few but typical clusters. If these incubated gametes (after being washed twice by centrifugation and resuspension in CONA-free medium) are combined with untreated gametes of their opposite sex, a clear-cut difference between the two sexes appears. Treated gynogametes of *C. eugametos* agglutinate with untreated androgametes to an undiminished degree, whereas absolutely no agglutination occurs in the reciprocal combination between treated androgametes and untreated gynogametes.

Such unilateral inhibition of one mating-type activity also exists within the two pairs of mating types of *C. moewusii* which are preliminarily designated as syngens I and II. The final designation of the two forms, which are sexually isolated by a nonmatching system of mating-type substances, will be made after their incompatibility mechanism has been analyzed in greater detail. The form which we call syngen I (Indiana Collection Nos. 96/97; Starr, 1964) is sexually compatible with *C. eugametos* (Indiana Collection Nos. 9/10); the latter's male sex (No. 9) being homologous to the (+) sex in *C. moewusii* syngen I (No. 96) (*cf.* Wiese, 1965). It is the (+) sex in *C. moewusii* syngen I which is sensitive to CONA. The sexual bipolarity for syngen II of *C. moewusii* (Indiana Collection Nos. 792/793, *cf.* Wiese and Metz, 1969) was fixed by declaring strain No. 792 as (+) sex because its cells continue the flagella beat and move the vis-à-vis pair after papilla fusion of the two mating partners [as does the (+) sex in *C. moewusii* syngen I (Lewin, 1952, 1954) and the male sex in *C. eugametos* (Wiese and Jones, 1963)]. Also in this taxon one sex only, and it is again the (+) sex, is sensitive to CONA.

The Sensitivity of the Mating Type Activities to 0.001% Concanavalin A and 0.1% Trypsin.

species sex	C. eugametos ♂	♀	C. moewusii syngen I +	−	C. moewusii syngen II +	−
Concanavalin A Sensitivity	S	R	S	R	S	R
Trypsin Sensitivity	R	S	R	S	R	S

FIGURE 1. The sensitivity of the mating-type activities to 0.001% concanavalin A (30 min incubation) and 0.1% trypsin (45 min incubation); S = sensitive; agglutinability entirely blocked or destroyed. R = resistant; no detectable diminution of the agglutinability. For further explanation see text.

This unilateral sensitivity of the agglutination mechanism to CONA in the three taxons is reciprocal to their unilateral sensitivity to 0.1% trypsin. Trypsin destroys the capacity to agglutinate in the female sex of *C. eugametos* and in the corresponding (−) sex of *C. moewusii* syngen I whereas the complementary sexes are trypsin-resistant (Wiese and Metz, 1969). Checking the two sexes of *C. moewusii* syngen II with 0.1% trypsin revealed also in this form, as in the other two taxons, only one of the two sexes sensitive, and it is again the (−) strain, *i.e.* that sex which is CONA-insensitive. As in *C. eugametos* and *C. moewusii* syngen I, the sexual inactivation of the gynogametes by trypsin is prevented by 0.05% trypsin inhibitor. Gynogametes of all three forms, sexually inactivated by trypsin, still possess their capacity to be adsorbed to CONA-coated sephadex.

All these effects of CONA in all three forms can be reversed by addition of 0.5% mannose. The various data for all three taxons are given in Figure 1.

The Agglutination Pattern of the Various Gamete Types with the Different CONA–isoagglutinin Complexes

		CONA–isoagglutinin Complexes					
		C. eugametos		C. moewusii syngen I		C. moewusii syngen II	
Test Gamete Type		♂	♀	+	–	+	–
C. eugametos	♂	O	A	O	A	O	O
	♀	O	O	O	O	O	O
C. moewusii syngen I	+	O	A	O	A	O	O
	–	O	O	O	O	O	O
C. moewusii syngen II	+	O	O	O	O	O	A
	–	O	O	O	O	O	O

FIGURE 2. The agglutination pattern of the various gamete types with the different concanavalin A—isoagglutinin complexes. The concanavalin-complexes of the taxons and sexes above were checked for their capacity to cause agglutination with the gamete types at the left; A = agglutination, O = no agglutination.

The normal sexual agglutination between andro- and gynogametes, and the two isoagglutinations caused by the isolated mating-type substances are not reversible by mannose.

From the supernatants of androgametes and of gynogametes, the sex-specific isoagglutinins are precipitated quantitatively by 0.1% CONA. The male and female precipitates, washed twice by centrifugation and resuspension in distilled water in order to remove unadsorbed CONA, act differently when combined with test gametes: in the complex formed with CONA, the male or (+) component has entirely lost its capacity to agglutinate gynogametes, whereas the precipitated female or (−) substance continues to extend its combining sites to androgametes. Precipitated at an excess of isoagglutinin, there is no detectable agglutination of either complex due to uncovered combining sites of CONA with the gametes of the homologous sex, or with both gamete types of the respectively incompatible species (Fig. 2).

DISCUSSION

The capacity to combine specifically with terminal sugars meeting certain specifications makes CONA a discriminating tracer for functions connected with polysaccharides or with the carbohydrate moiety of complex molecules. The mating-type reaction in isogamous *Chlamydomonas* species involves an instantaneous agglutinative adhesion between sexually different gametes. According to the analyses of the isoagglutinins which represent isolated mating-type substances (*cf.* Wiese, 1965, 1969), the contact function is inherent to components of glycoprotein nature (Förster, Wiese and Braunitzer, 1956; Wiese, 1961, 1965, 1967, 1969). Whatever the type of surface to surface interaction may be, the responsible contact mechanism is distinguished by the promptness and specificity of its reaction. A special requirement exists for the presence of bivalent cations (Ca^{++}, Mg^{++}) (Lewin, 1954; Tsubo, 1961; Wiese and Jones, 1963). Details of the mating-type reaction reveal that the agglutination mechanism in dioecious *Chlamydomonas* species must operate on the basis of a complementarity between sex-specific components, must be located on or associated with the flagella tip, and must be biosynthesized or set into action during gametogenesis (*cf.* Wiese, 1969). In addition, the mechanism must be responsible for the absolute species specificity of gamete contact. In accordance with the general concept on sex cell contact as an antigen-antibody like relation (*cf.* Metz, 1967), we assume that the bipolar sexual difference between the two gamete types is based upon a complementarity between components standing in mutual receptor relationship. In *Chlamydomonas,* we encounter the problem that the two isoagglutinins do not neutralize each other *in vitro* (Wiese and Wiese, 1965).

The sexual complementarity between andro- and gynogametes can be unilaterally addressed by CONA: combination of CONA with the terminal sugars on the flagella surface of androgametes (*i.e.*, with the mating-type substances *in situ*) and on the isolated male mating-type substance (the male isoagglutinin) totally removes the agglutinability. A corresponding complex formation with CONA also occurs with the female components as documented by the CONA-induced isoagglutination of gynogametes, by the specific attachment of gynogametes to CONA-coated sephadex, and by the precipitation of the female isoagglutinin. In the female sex, however, the combination of CONA with the terminal sugars does not interfere with the component's capacity to agglutinate. The female receptor sites involved in sexual agglutination, are not identical with the CONA-adsorbing sites, and, in addition, both areas do not overlap in a manner that the sexual receptor site is sterically blocked by the CONA-adsorption to the sugars. In accordance with this, tryptically inactivated gynogametes still adsorb CONA. This interpretation of the CONA-effect requires additional confirmation of the special role ascribed to the sugars, since the inactivation of one sex might be caused by an unspecified complex formation of CONA with the contact component due to its polyelectrolyte nature.

In both sexes of all three taxons, the complex formed with CONA can be dissociated by mannose restoring in all three types of androgametes the unaltered capacity to agglutinate. Of the individual sugars which are the same in all six isoagglutinins (rhamnose, xylose, arabinose, galactose and mannose), only mannose splits the CONA-complex with the mating-type substances. Mannose was identified by Dr. P. Weber, Department of Biochemistry, State University of New York at Buffalo. The proper mating-type reaction between andro- and gynogametes, and the two isoagglutinations caused by a form's isoagglutinins are not prevented or reversed by mannose.

The reciprocal, unilateral interference of trypsin with the mating-type reaction does not necessarily indicate the direct inactivation of a proteinaceous receptor. Further characterization by blocking protein-specific functional groups and more data on the chemical composition and on the functional architecture of the female isoagglutinin are needed to confirm the protein nature of the female receptor site.

In addition to the sex-discriminating effect of CONA, its reaction with *Chlamydomonas* gametes demonstrates how an interaction between appropriate macromolecules in the cell's surface can account for a cell contact as instantaneous as is typical for the mating-type reaction. A compound such as CONA even shares those structural properties of the mating-type substances which provide them, in their isolated state, with the capacity to cause isoagglutination. The data suggest that, at normal sex cell contact, a proteinaceous component on one gamete type with combining sites similar to those of CONA might interact with a complementary sugar-containing component on the other gamete's surface.

A common dependency of the interaction of CONA with sugar-containing macromolecules (Agrawal and Goldstein, 1968) and of the mating-type reaction on bivalent cations further supports the parallelism between both events. On the basis of a similar relation, Rybak and Burstein (1960) concluded that, since fertilizin agglutinates with lipoproteins in the sera of mammals in the presence of Ca^{++}, a lipid component on the sperm might interact with the fertilizin of the egg in the Ca-dependent gamete contact at sea urchin fertilization. For *Chlamydomonas* (*cf.* Wiese, 1961), the further analysis of the male mating-type substance and of the effect of glycosidases upon androgametes will decide whether the two Ca^{++} actions need exclude one another. In addition, Ca^{++} in the observed concentrations may well be engaged in functions other than direct contact making (*cf.* Köhler, 1956).

In addition to its promptness, a most impressive feature of sex cell contact is the absolute specificity governing this event in a manner that no trial and error attempts are initiated between gametes of the same sex or between incompatible gamete types. Either the contact ensues instantaneously—within part of a second, a certain cell density provided—or not at all. Affecting the bipolar agglutination mechanism in each form unilaterally, neither CONA nor trypsin observes the incompatibility barrier between the three taxons, revealing that some basic sexual surface bipolarity exists in each form which is alternatively eliminated by the two agents. The features which condition the species specificity of the agglutination mechanism must be overimposed on this bipolarity, modifying it in a manner that the bipolarity comes to bear only within the matching pattern of the right system.

SUMMARY

Concanavalin A (CONA) isoagglutinates the gametes within each sex of *Chlamydomonas eugametos*, *C. moewusii* syngen I, and *C. moewusii* syngen II by a typical agglutinative adhesion of the flagella tips. Incubation of gametes with

lower CONA-concentrations which do no longer cause isoagglutination, effects blockage of the mating-type activity in only one of the two sexes, the other one being unaffected. In the three taxons investigated, it is always the androgametes which are sensitive to CONA. In addition, CONA immobilizes the cells after a short period of time. The CONA-induced isoagglutinations of both gamete types, the immobilization, and the unilateral blocking of one mating-type activity can be reversed entirely by mannose. In the three taxons, the unilateral sensitivity of the mating-type reaction to CONA is reciprocal to a unilateral sensitivity to 0.1% trypsin. Gynogametes, sexually inactivated by trypsin, still interact with CONA. In each of the three taxons, CONA precipitates both sexual isoagglutinins but only one of them, that of the CONA-insensitive sex, in active form. The data are discussed as to their bearing on the nature of the initial contact mechanism between sex cells.

LITERATURE CITED

Agrawal, B. B. L., and I. J. Goldstein, 1968. Protein-carbohydrate interaction. XV. The role of bivalent cations in concanavalin A—polysaccharide interaction. *Canad. J. Biochem.*, **46**: 1147–1150.

Baker, P. M., and L. Wiese, 1968. Immunological analysis of the mating type reaction in *Chlamydomonas*. *J. Phycol.*, **4**: 3 (Abstract).

Doyle, R. J., E. E. Woodside and C. W. Fishel, 1968. Protein-polyelectrolyte interactions. The concanavalin A precipitin reaction with polyelectrolytes and polysaccharide derivates. *Biochem. J.*, **106**: 35–40.

Förster, H., L. Wiese and G. Braunitzer, 1956. Über das agglutinierend wirkende Gynogamon von *Chlamydomonas eugametos*. *Z. Naturforsch.*, **11b**: 315–317.

Goldstein, I. J., and R. N. Iyer, 1966. Interaction of concanavalin A, a phytohemagglutinin, with model substrates. *Biochim. Biophys. Acta*, **121**: 197–200.

Goldstein, I. J., and L. L. So, 1965. Protein-carbohydrate interaction. III. Agar gel diffusion studies on the interaction of concanavalin A, a lectin isolated from jack bean, with polysaccharides. *Arch. Biochem. Biophys.*, **111**: 407–414.

Goldstein, I. J., C. E. Hollerman and J. M. Merrick, 1965. Protein-carbohydrate interaction. I. The interaction of polysaccharides with concanavalin A. *Biochim. Biophys. Acta*, **97**: 68–76.

Goldstein, I. J., C. E. Hollerman and E. E. Smith, 1965. Protein-carbohydrate interaction. II. Inhibition studies on the interaction of concanavalin A with polysaccharides. *Biochem.*, **4**: 876–883.

Köhler, K., 1956. Entwicklungsgeschichte, Geschlechtsbestimmung und Befruchtung bei *Chaetomorpha*. *Arch. Protistenk.*, **101**: 223–268.

Lewin, R. A., 1952. Studies on the flagella of algae. I. General observations on *Chlamydomonas moewusii* Gerloff. *Biol. Bull.*, **102**: 74–79.

Lewin, R. A., 1954. Sex in unicellular algae, pp. 100–133. *In:* D. H. Wenrich, Ed., *Sex in Microorganisms*. American Association Advancement of Science Washington, D. C.

Metz, C. B., 1967. Gamete surface components and their role in fertilization, pp. 163–224. *In:* C. B. Metz and A. Monroy, Eds., *Fertilization. Comparative Morphology, Biochemistry and Immunology*. Academic Press, New York.

Rybak, B., and Burstein, M., 1960. Une combinaison chimique de la fertilisine. *Experientia* **16**: 216.

So, L. L., and I. J. Goldstein, 1967. Protein-carbohydrate interaction. IX. Application of the quantitative hapten inhibition technique to polysaccharide-Concanavalin A interaction. Some comments on the forces involved in Concanavalin A-polysaccharide interaction. *J. Immunol.*, **99**: 158–163.

So, L. L., and I. J. Goldstein, 1968. Protein-carbohydrate interaction. XX. On the number of combining sites on concanavalin A. *Biochim. Biophys. Acta*. **165**: 398–404.

STARR, R. C., 1964. The culture collection of algae at Indiana University. *Amer. J. Bot.*, **51**: 1013–1044.

SUMNER, J. B., AND S. F. HOWELL, 1936. The identification of the hemagglutinin of the jack bean with concanavalin A. *J. Bacteriol.*, **32**: 227–237.

TSUBO, Y., 1961. Sexual reproduction of *Chlamydomonas* as affected by ionic balance in the medium. *Botan. Mag. (Tokyo)*, **74**: 442–448.

WIESE, L., 1961. Gamone. *Fortschr. Zool.*, **13**: 119–145.

WIESE, L., 1965. On sexual agglutination and mating-type substances (gamones) in isogamous heterothallic Chlamydomonads. I. Evidence of the identity of the gamones with the surface components responsible for sexual flagellar contact. *J. Phycol.*, **1**: 46–54.

WIESE, L., 1967. The mating-type substance of the male sex in *Chlamydomonas eugametos*. *J. Phycol.*, **3**: 9 (Abstract).

WIESE, L., 1969. Algae, pp. 135–188. *In:* C. B. Metz and A. Monroy, Eds., *Fertilization. Comparative Morphology, Biochemistry,* and *Immunology*. Academic Press, New York.

WIESE, L., AND R. F. JONES, 1963. Studies on gamete copulation in heterothallic Chlamydomonads. *J. Cell. Comp. Physiol.*, **61**: 265–274.

WIESE, L., AND C. B. METZ, 1969. On the trypsin sensitivity of gamete contact at fertilization as studied with living gametes in *Chlamydomonas*. *Biol. Bull.*, **136**: 483–493.

WIESE, L., AND W. WIESE, 1965. On sexual agglutination and mating-type substances in *Chlamydomonas*. *Amer. J. Botan.*, **52**: 621. (Abstract).

Reference: *Biol. Bull.*, **138**: 96–108. (February, 1970)

OSMOREGULATORY CAPABILITIES IN ISOPODS: *LIGIA OCCIDENTALIS* AND *LIGIA PALLASII*[1]

WILFRED J. WILSON

Department of Zoology, San Diego State College, San Diego, California 92115

The shore isopods (genus *Ligia*) are crustaceans which are thought to be in transition between water and land environments. They are physiologically adapted for submergence in varying concentrations of sea water and for foraging in a terrestrial environment which shows wide fluctuations in temperature, but to survive they must alternate periods in both situations.

The experiments reported in this study were undertaken to determine the effects of temperature, sex, size, and combinations of these variables, on the ability of *Ligia occidentalis* Dana and *L. pallasii* Brandt to regulate osmotically when exposed to various concentrations of sea water.

Early osmoregulatory studies on isopods were hampered by a lack of methods for studying the minute volumes of body fluid found in small individual animals. Barnes (1932) studied the response of *Ligia baudiniana* in media ranging from distilled water to 100% sea water. He found less survival in distilled water and assumed that *L. baudiniana* was an osmoconformer. Bateman (1933), using vapor pressure methods, pooled the haemolymph of a group of *L. oceanica* living on moist seaweed and found it to be slightly hyperosmotic to 100% sea water.

With the modifications of micro-cryoscopic methods described by Ramsay (1949), recent investigators have measured haemolymph osmotic pressures of individual animals. Using a limited number of individuals at 8° C, Parry (1953) found the haemolymph of *Ligia oceanica* submerged in various sea water concentrations to be hyperosmotic to sea water concentrations of less than 100% and isosmotic to concentrations of more than 100%. Riegel (1959a, b) found that some sphaeromid isopods regulate hyperosmotically in dilute concentrations of sea water. He proposed the elevation of two subspecies of *Gnorimosphaeroma oregonensis* to species level on the basis of differences observed in their hyperosmotic regulation as well as on the basis of morphological and ecological differences between them. Lockwood (1959, 1960) found the haemolymph of the fresh water isopod *Asellus aquaticus* to be hyperosmotic in the range from distilled water to about 50% sea water. He found osmoconformity in concentrations of more than 50% sea water.

The role of temperature in osmoregulation has not been fully explored. In his experiments on sphaeromid isopods, Riegel (1959a) demonstrated that haemolymph concentrations dropped more rapidly at 16° C than at 5° C when animals were submerged in fresh water.

Todd (1963) reports that season and temperature cause *Ligia oceanica* and *Idotea granulosa* (a marine isopod) to modify their hyperosmotic regulation in salinities below 100% sea water. Todd collected *L. oceanica* and *I. granulosa* in winter and summer months and measured their osmotic responses at 5° and 15° C. She found that summer animals of both species regulated less than did winter animals. In salinities of more than 50% sea water, animals adapted at 5° C regulated more than did animals adapted at 15° C. At lower salinities she could not demonstrate the influence of temperature on the ability of these animals to regulate their haemolymph osmotic pressure. Weight changes corresponding with changes in the haemolymph concentrations were considered to be negligible, although apparently they were not measured.

---

[1] A portion of a thesis presented in partial fulfillment of the requirements for the Ph.D. degree at the University of California, Davis.

MATERIALS AND METHODS

Ligia occidentalis was obtained from a rocky bay shore one-half mile north of Stinson Beach, California. Male and female adults ranging in weight from 0.2 to 0.4 g were collected.

Ligia pallasii was collected from sandstone cliffs, some 15 feet above the high tide mark, on the open coast near Moss Beach, California. Male adults from 0.7 to 1.0 g and female adults from 0.3 to 0.55 g were obtained.

Both species were stored separately in filtered aerated sea water ($\triangle e = 2.08$) at 12° C. (The symbol $\triangle e$ refers to the freezing point depression in degrees centigrade of the external environmental fluid and the symbol $\triangle i$ to that of the internal body fluid.) They were transferred to controlled temperature rooms at least 24 hours prior to immersion in the experimental external media. A high temperature (22° ± 1° C) and a low temperature (12° ± 1° C) were chosen to approximate the seasonal extremes of sea water temperature encountered in areas of collection.

The solutions used as experimental external media ranged from 25% ($\triangle e = 0.54$) to 175% sea water ($\triangle e = 3.67$). All experimental concentrations were obtained by evaporation and then dilution with distilled water. One hundred percent sea water was maintained at 36‰ ($\triangle e = 2.08$). This closely correlated with surface salinity values from Moss Beach and Stinson Beach for the months during which the animals were collected. The concentrations of all experimental media were checked by a chlorinity titration method (Giese, 1962).

The animals were placed in the filtered, aerated experimental solutions for a period of 48 hours, since preliminary experiments demonstrated that internal osmotic pressure ($\triangle i$) reached the steady state by that time. During this period the animals were starved, kept in darkness, and maintained in groups of 10 with a minimum of 100 ml of experimental solution per animal at each salinity and temperature.

The haemolymph was drawn directly from the heart region using a capillary tube with a 0.4 mm bore. A 2 to 3 mm column of fluid was collected and centered in a 3 cm long tube. Both ends of the tube were then plugged with plasticine.

Osmotic concentrations of the haemolymph samples were determined by a modification of the methods of Ramsay (1949) and Gross (1954) for determining freezing point depressions. These methods are accurate to 0.02° C.

Tubes containing haemolymph and tubes containing solutions of NaCl with known melting points of 0°, −1°, −2°, and −3° C were positioned on a plastic grooved holder and placed on dry ice. After freezing, the samples on the plastic holder were placed in an insulated chamber containing ethanol at approximately −5° C. To raise the temperature gradually, the ethanol was stirred continuously with an air stirrer. Visibility of the ice crystals in the samples was improved by using a magnifying glass and two sheets of polaroid film. One sheet of film was positioned near the observation glass, and the other was placed at the bottom of the plastic container. The time of melting was determined by taking the midpoint in the melting process. If any doubt existed, second and third readings were taken.

The melting times of the samples with known melting points were then plotted against the melting times of samples with unknown melting points. Assuming melting rates to be linear over one degree increments and melting points equal to freezing points, unknown melting points (as freezing point depressions in degrees centigrade) were found by interpolating the time between known melting points.

Any significant effect on the osmotic concentration of the haemolymph due to species, salinity, temperature, or interactions between these factors was determined by the analysis of variance (Dixon and Massey, 1969).

RESULTS

Ligia occidentalis

Ecology and behavior. Ligia occidentalis was observed under and among rocks in three intertidal regions of central California: on the outer rocky coast of the Pacific Ocean, near brackish waters in San Francisco Bay, and near the relatively fresh water environs of the lower Sacramento River. The shore areas and water temperatures of these areas vary tremendously. In those shore areas where temperature fluctuations are greatest, the nearby water environments have the lowest salt concentrations.

L. occidentalis is a scavenger which is normally quite active during cool, humid periods. It can also forage to a limited extent during hot, dry periods by moving rapidly from place to place and can thereby avoid spending significantly long periods in hostile environments.

Osmotic balance. At 12° C in experimental media of 75%, 100%, 125%, 150% and 175% aerated sea water, *Ligia occidentalis* lived submerged for over six weeks, whereas in 50% sea water half of the animals died within five days. At 12° and 22° C in 25% sea water all animals were dead within 48 hours; therefore, osmotic concentrations of their haemolymph were not measured. At 22° C in 50% sea water and above, none survived more than five days.

One hundred and forty determinations of freezing point depressions were made on the haemolymph of animals which had been collected in summer months and adapted to experimental salinities and temperatures for 48 hours. As indicated in Figure 1, the data clearly show that at lower concentrations of sea water, the haemolymph has significantly lower osmotic concentration (salinity alone, $F = 168.72$, $P < 0.01$). In salinities above 100% sea water, *Ligia occidentalis* was hypoosmotic, and below 100% sea water it was hyperosmotic. The difference between internal and external osmotic pressures increased as the external concentration was raised or lowered from 100% sea water.

At 175% sea water concentrations, significant differences in osmotic concentration were demonstrated between animals adapted at 12° C and those adapted

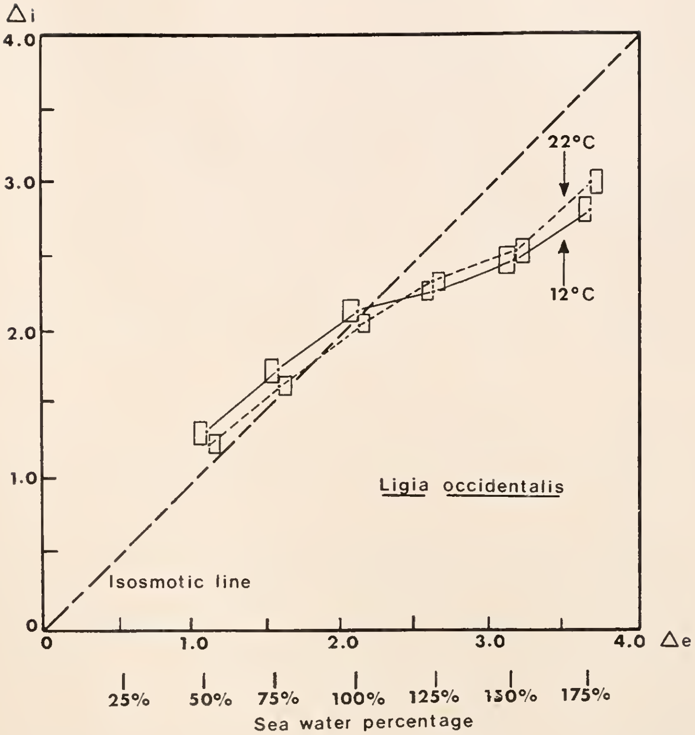


FIGURE 1. The relation of the osmotic concentration of the haemolymph (Δ*i*) of *Ligia occidentalis* to the concentration of the medium (Δ*e*) at 12° C (continuous line) and 22° C (broken line). Two times the standard error has been plotted on each side of the means for internal osmotic concentrations at each temperature-salinity concentration.

at 22° C ($P < 0.05$, Fig. 1). In other temperature-salinity combinations, significant differences could not be demonstrated statistically ($P > 0.05$). It was found that throughout the series of experimental salinities, animals adapted at 12° C showed significantly different osmotic concentrations of haemolymph from those adapted at 22° C (temperature-salinity interaction, $F = 3.39$, $P < 0.01$). At both temperatures, however, *Ligia occidentalis* is a slight hyperosmotic regulator in concentrations of sea water of less than 100% and a hypoosmotic regulator in concentrations above 100% (Fig. 1).

Effects of size and sex. From each temperature-salinity medium, an equal number of male and female animals between 0.2 and 0.4 g were selected for measurements of osmotic concentration.

Sex, weight, and internal osmotic pressure of animals in 150%, 100%, and 50% sea water at 12° and at 22° C were determined. No significant differences in regulatory abilities in relation to weight or sex could be demonstrated.

Rates of osmotic and weight change. Animals adapted to 150% sea water at 12° and 22° C were transferred to 50% sea water at the same temperatures. Internal osmotic concentrations and weights were determined at 0, 2, 4, 8, 16, 24, and 48 hours. These measurements are plotted in Figures 2 and 3.

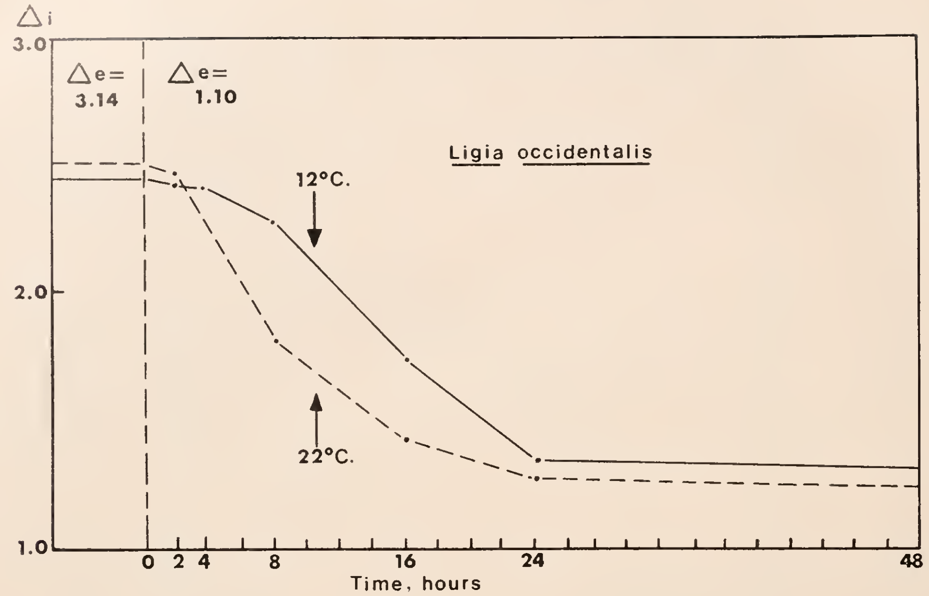


FIGURE 2. Changes in internal osmotic pressure (Δ*i*) of *Ligia occidentalis* when external osmotic pressure (Δ*e*) is reduced from 3.14 (150% sea water) to 1.10 (50% sea water) at 12° C and at 22° C during 48 hours.

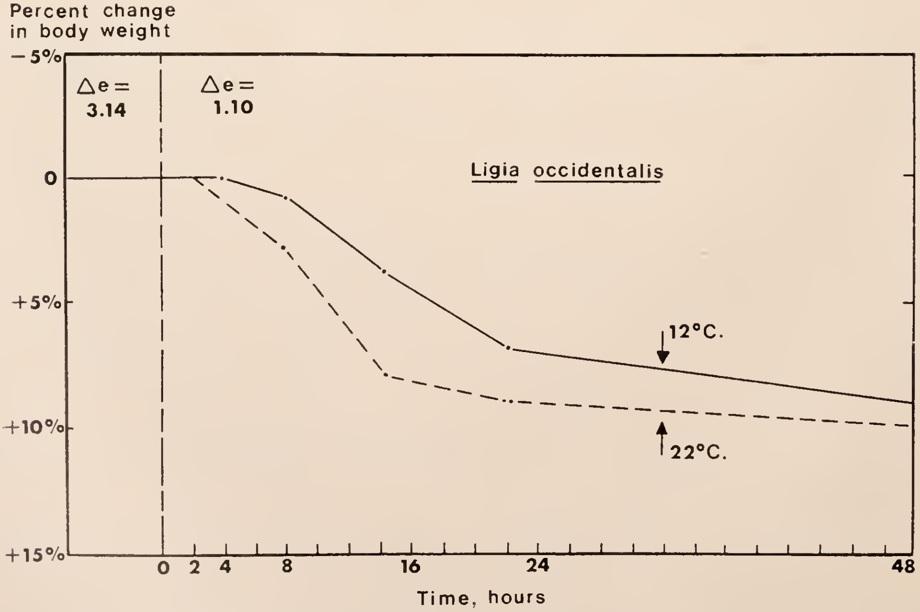


FIGURE 3. Changes in body weight as per cent of initial body weight of *Ligia occidentalis* when external osmotic pressure (Δ*e*) is reduced from 3.14 (150% sea water) to 1.10 (50% sea water) at 12° C and 22° C during 48 hours.

Comparing rates of change in internal osmotic concentrations, a significant difference ($P < 0.01$) was demonstrated between 12° and 22° C. The internal osmotic concentration of the haemolymph of animals adapted to 22° C came to equilibrium at a faster rate than that of animals adapted to 12° C. The internal osmotic concentration dropped from $\Delta i = 2.46$ to $\Delta i = 1.31$ at 12° C in 48 hours and from $\Delta i = 2.50$ to $\Delta i = 1.25$ at 22° C in 48 hours (Fig. 2).

The body weight of animals adapted to 22° C also came to equilibrium at a significantly faster rate than did the weight of animals adapted to 12° C ($P < 0.01$). The mean percent body weight changes 48 hours after transfer to 50% sea water were +9% at 12° C and +10% at 22° C ($P > 0.05$).

No differences in rates of change of osmotic concentrations and of body weights could be demonstrated between male and female specimens of *Ligia occidentalis*.

Ligia pallasii

Ecology and behavior. *Ligia pallasii* was found in large colonies on the central California coast in regions where temperature and desiccating conditions are not extreme. These animals live 5 to 20 feet above high tide marks in crevices located along cliffs.

The body proportions of the adult animals exhibit a marked sexual dimorphism. The males are large and broad and tend to group themselves so as to form shields for the females and immature males. *L. pallasii* is a slow-moving scavenger, and thus restricts its foraging activities to cool, humid periods.

Osmotic balance. At 12° C in experimental media of 50%, 75%, 100%, 125%, 150%, and 175% sea water, *Ligia pallasii* lived submerged for over six weeks, whereas in 25% sea water half the animals died within eight days. At 12° and 22° C in distilled water and in tap water all animals died within 12 hours; therefore, osmotic concentrations of their haemolymph were not measured. At 22° C in 25% sea water and above, none survived more than five days.

One hundred and forty determinations of freezing point depressions were made on the haemolymph of animals which had been collected in summer months and adapted to experimental salinities and temperatures for 48 hours. As indicated in Figure 4, it is obvious that at lower concentrations of sea water, the haemolymph shows significantly lower osmotic concentration (salinity alone, $F = 97.23$, $P < 0.01$). In salinities above 100% sea water, *Ligia pallasii* was hypoosmotic and below 100% sea water it was hyperosmotic. The difference between internal and external osmotic pressures increased as the external concentration was raised or lowered from 100% sea water.

At 25% and 100% sea water concentrations, significant differences in osmotic concentrations were demonstrated between animals adapted at 12° C and those adapted at 22° C ($P < 0.05$). In other temperature-salinity combinations, significance could not be demonstrated ($P > 0.05$). It was found that throughout the series of experimental salinities, animals adapted at 12° C showed significantly different osmotic concentrations of haemolymph from those adapted at 22° C (temperature-salinity interaction, $F = 3.39$, $P < 0.01$). At both temperatures, however, *Ligia pallasii* is a good hyperosmotic regulator in concentrations of sea water of less than 100% and a good hypoosmotic regulator in concentrations above 100% (Fig. 4).

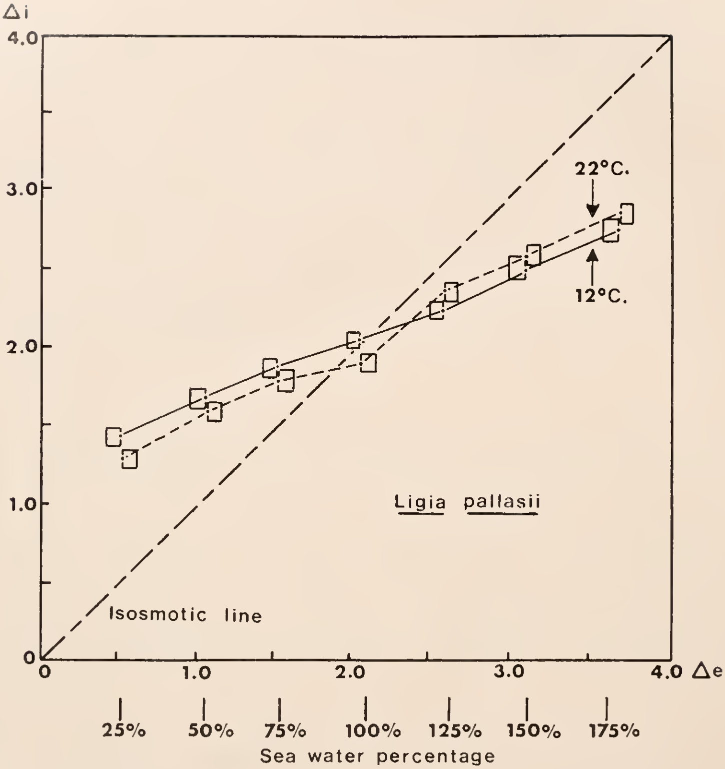


FIGURE 4. The relation of the osmotic concentration of the haemolymph (Δ*i*) of *Ligia pallasii* to the concentration of the medium (Δ*e*) at 12° C (continuous line) and 22° C (broken line). Two times the standard error has been plotted on each side of the means for internal osmotic concentrations at each temperature-salinity concentration.

Effects of size and sex. From each temperature-salinity medium, an equal number of male animals between 0.7 and 1.0 g and female animals between 0.3 and 0.55 g were selected for measurements of osmotic concentration. Sex, weight, and internal osmotic pressure of animals in 150%, 100%, and 50% sea water at 12° and at 22° C were determined. No significant differences in regulatory abilities in relation to weight or sex could be demonstrated.

Rates of osmotic and weight change. Animals adapted to 150% sea water at 12° and 22° C were transferred to 50% sea water at the same temperatures. Internal osmotic concentrations and weights were determined at 0, 2, 4, 8, 16, 24, and 48 hours. These measurements are plotted in Figures 5 and 6.

Comparing rates of change in internal osmotic concentrations, a statistically significant difference ($P < 0.01$) was demonstrated between 12° and 22° C. The internal osmotic concentration of the haemolymph of animals adapted to 22° C came to equilibrium at a faster rate than that of animals adapted to 12° C. The internal osmotic concentration dropped from $\Delta i = 2.50$ to $\Delta i = 1.67$ at 12° C in 48 hours and from $\Delta i = 2.59$ to $\Delta i = 1.61$ at 22° C in 48 hours (Fig. 5).

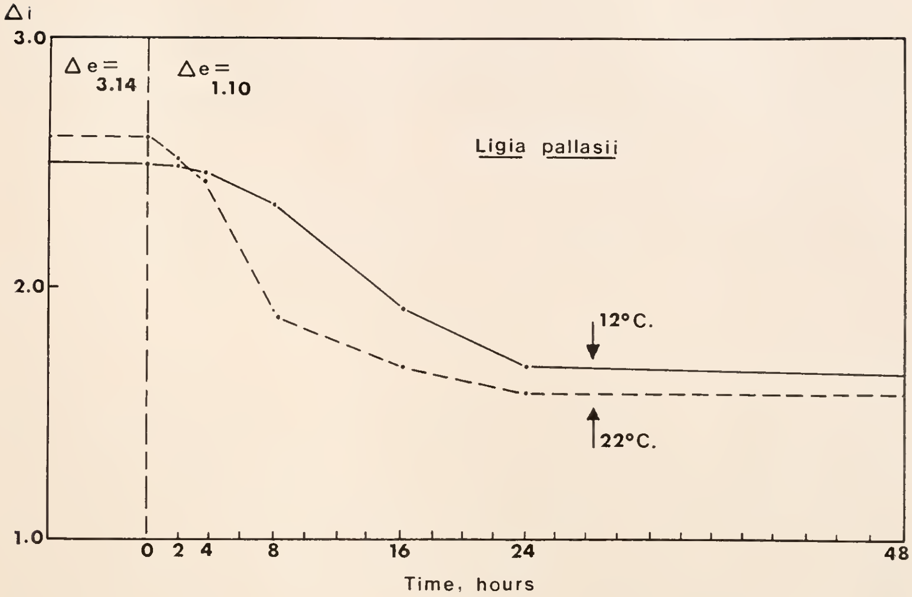


FIGURE 5. Changes in internal osmotic pressure (Δ*i*) of *Ligia pallasii* when external osmotic pressure (Δ*e*) is reduced from 3.14 (150% sea water) to 1.10 (50% sea water) at 12° C and 22° C during 48 hours.

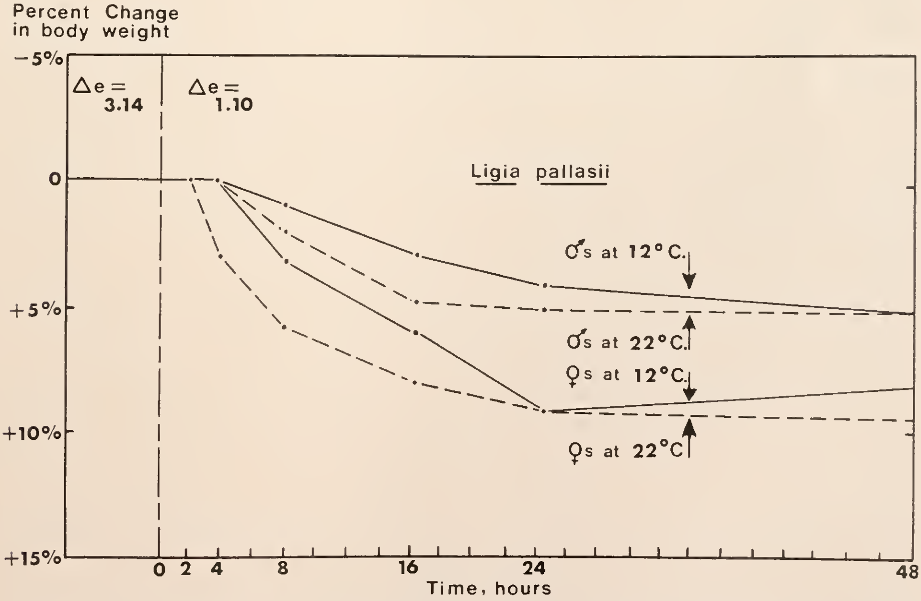


FIGURE 6. Changes in body weight as per cent of initial body weight of *Ligia pallasii* when external osmotic pressure (Δ*e*) is reduced from 3.14 (150% sea water) to 1.10 (50% sea water) at 12° C and 22° C during 48 hours.

The body weight of animals adapted to 22° C also came to equilibrium at a significantly faster rate than did the weight of animals adapted to 12° C ($P < 0.01$). The mean percent body weight changes 48 hours after transfer to 50% sea water were significantly different between male and female animals ($P < 0.05$). Male animals gained 5% both at 12° and at 22° C and females gained 8% at 12° and 9% at 22° C (Fig. 6).

No differences in rates of change of osmotic concentrations could be demonstrated between male and female specimens of *Ligia pallasii*. At both temperatures male animals gained a smaller per cent of body weight than did female animals.

DISCUSSION

General osmoregulatory response

The osmoregulatory responses shown by *Ligia occidentalis* and *L. pallasii* correlate well with their behavior and ecology. In comparing the two species, one finds that they both can live in relatively cool and foggy open-coast areas where temperatures and desiccating conditions are not extreme. However, *L. occidentalis* can extend its range into drier, terrestrial regions. It appears to do this by moving faster and by alternating its activity between periods of foraging in dry environments and periods of exposure to wet conditions of variable salinity where it can replace any water which may have been lost by evaporation. *L. pallasii* moves more slowly and thus must spend longer periods in the wetter hyposaline environment and less time in the drier environment. It hyperosmoregulates and survives better than does *L. occidentalis* in the wetter hyposaline environment.

When osmoregulatory responses of *Ligia occidentalis* and *L. pallasii* are compared with the responses of *L. oceanica*, reported by Parry (1953) and Todd (1963), it is obvious that interspecific similarities and differences do occur. All three species show hyperosmotic regulation when immersed in sea water concentrations of less than 100%. This response maintains the proper water balance of tissues at the expense of metabolic work. This response is of particular importance to *L. pallasii*, which in its natural environment spends prolonged periods in hyposaline conditions. The hypoosmotic regulatory response in concentrations of more than 100% sea water enables the two species in this study to resist water loss. Parry (1953) found *L. oceanica* to be an osmoconformer in salinities of more than 100% sea water indicating that possibly more water can be lost in hypersaline conditions than is the case for *L. occidentalis* and *L. pallasii*.

In extreme drying conditions, *Ligia oceanica* had an internal osmotic pressure (Δi) of 3.48 (Parry, 1953) and *L. occidentalis* 3.25 (as measured by the author). These measurements indicate an increase in internal osmotic pressure due to a loss of water to a terrestrial environment. The study of Miller (1938) demonstrated that *L. occidentalis* and *L. pallasii*, when exposed to continuous low relative humidity levels, do not survive as well as they do at higher levels. From these studies it appears that *Ligia* cannot withstand prolonged periods of dryness and must periodically obtain water in another environment.

In low concentrations of sea water all species of *Ligia* studied thus far have osmoregulatory capabilities which have little survival value after prolonged exposure. Various degrees of regulation in species of *Ligia* modify, but do not change, the basic response to this extreme.

Each species of *Ligia* has its own osmoregulatory requirements to meet when temporarily dealing with a severe environment. To survive, all species must alternate varying periods of dryness with varying periods of wetness, both of which may be fatal in extreme.

Temperature influence

The osmoregulatory responses of *Gammarus duebeni* at 7° and 21° C (Kinne, 1952, 1953) are similar, except at low salinities, to those found for *Ligia occidentalis* and *L. pallasii* (Figs. 1 and 5). Nemenz (1960) found that the larvae of the brine fly, *Ephydra cinerea*, osmoregulated less at high temperatures. His salt concentrations were many times greater than those in which *L. occidentalis* and *L. pallasii* were placed.

Osmoregulation appears to be influenced by temperature in both *Ligia occidentalis* and *L. pallasii* (Figs. 1 and 4). The similarity between the osmotic pressure of haemolymph and that of the external environment is greater at 22° C than it is at 12° C. This implies less osmotic regulation at the higher temperature. It can be assumed that the maintenance of an osmotic gradient requires metabolic work and that at a higher temperature the metabolic rate would increase (a Q10 of 2 or 3 can be expected). Cell permeability should also increase at higher temperatures, thus increasing the metabolic work required to maintain an osmotic gradient. Potts and Parry (1964) estimate that the total osmotic work in *Nereis diversicolor* submerged in sea water concentrations of less than 50% at 25° C is 0.02% to 2.5% of the total metabolic work of the animals. Environmental extremes of temperature were thought not to influence these percentages. These measurements suggest that osmotic stress does not drain significant energy from other metabolic processes.

The results of these experiments with *Ligia occidentalis* and *L. pallasii* agree with the observations of Panikkar (1940) on the prawns *Leander serratus* and *Palaemonetes varians*, with those of Lockwood (1960) on the isopod, *Asellus aquaticus*, and with those of Todd (1963) on the isopods *L. oceanica* and *Idotea granulosa*. In hyposaline conditions these species maintained a higher haemolymph concentration at low temperatures than they did at higher temperatures. Panikkar concluded from his results that warmer regions would favor colonization of brackish water by marine organisms because haemolymph is less hyperosmotic to the environment at higher temperatures. This statement has been criticized by Robertson (1960) because it assumes that animals can adjust their metabolisms to optimal efficiency at different internal osmotic pressures. Robertson notes that there is no direct evidence for this assumption.

The effects of sex and body weight

The ability of *Ligia occidentalis* and *L. pallasii* to maintain levels of osmoregulatory response is independent of body weight and sex. Todd (1963) had similar results using *L. oceanica*. These results on *Ligia* are to be expected since adult and young individuals of these species apparently spend their lives in the same environment.

Miller (1938) noted marked sexual dimorphism in *Ligia pallasii* and found that expanded flattened males form a shield to protect females and smaller males from exposure to low relative humidities. The survival time of shielded animals was twice that of non-shielded animals. The observation in this study that males gain a lesser per cent of initial body weight than do females, when submerged in reduced concentrations of sea water, may indicate a greater survival in adverse conditions (Fig. 6).

Indications of water exchange

Edney (1952, 1953) measured rectal temperatures of *Ligia oceanica* to determine the influence of transpiration in lowering body temperature below ambient temperature. He found a 7° C reduction that he attributed to evaporation. Since water is used to enable *Ligia* to occupy temporarily a hot hostile environment, water must be lost and body weight reduced. Experiments, which increased the haemolymph concentration by desiccation in a terrestrial environment, by the author on *L. occidentalis* and *L. pallasii* and by Parry (1953) on *L. oceanica* indicate at least some of the water lost was osmotically active water.

When *L. occidentalis* and *L. pallasii* were adapted to 150% sea water and then transferred to 50% sea water, animals at 12° C came to a steady internal osmotic pressure later and gained weight more slowly than did those at 22° C. Riegel (1959a) could not demonstrate weight changes in sphaeromid isopods at 16° C in similar experiments, although weight changes were noted at lower temperatures.

Riegel (1959a) attributes major changes in internal osmotic pressure to active secretion and absorption of salts. This may be partially true for *Ligia*, but it is apparent that when weight changes up to 10% of initial body weight are observed, dilution or concentration of haemolymph by water movement is the major method for change in internal osmotic pressure (Figs. 3 and 6).

Differential rates of change of internal osmotic pressure may be a limiting factor to distribution of *Ligia*. Short periods of submergence in extreme concentrations of sea water at high temperatures may raise or lower their internal osmotic pressure sufficiently to harm the animals. These same short exposures at lower temperatures may not prove harmful.

Sites of water and salt exchange

The success of arthropods in terrestrial and fresh water environments is due to the relative impermeability of the cuticle. In isopods the primary sites for water and ion exchange are believed to be the mouth, anus, gut, and the moist respiratory pleopod region. Oral drinking has been demonstrated by Lockwood (1962) in the isopods *Oniscus asellus, Porcellio scaber,* and *Armadillidium vulgare.* Edney and Spencer (1955) demonstrated, by using eosin marked media, that *Ligia oceanica* takes up water primarily through the anus. Parry (1953) states that the most likely sites for active uptake of water are the pleopods and uropods in *L. oceancia.* Although studies demonstrating movements of water through the gut region and pleopods of isopods have not been reported, Nemenz (1960) found the primary site of water transfer in the brine fly, *Ephydra cinerea,* to be the gut, and Croghan (1958) found indications of ion exchange at the respiratory branchiae of the brine shrimp, *Artemia salina.*

I should like to express my appreciation to Dr. Arthur C. Giese for providing help with techniques and for inspiration, and to Drs. George W. Salt, Charles L. Judson, and Jurgen H. Jacobs for many helpful suggestions. I am especially grateful to Dr. Milton A. Miller for the guidance and criticism which have contributed to this study.

SUMMARY

The influence of salinity, temperature, size, and sex on the osmoregulatory response of the seashore isopods *Ligia occidentalis* and *L. pallasii* was determined, and the environmental relationships are discussed.

1. *Ligia occidentalis* and *L. pallasii* have similar osmoregulatory responses to changing salinities at 12° and 22° C. In salinities of more than 100% sea water, both were hypoosmotic. The difference between internal and external osmotic pressures increased as the external concentration was raised or lowered from 100% sea water.
2. The difference between internal and external osmotic pressures at each temperature-salinity combination was less at 22° than at 12° C indicating less osmotic regulation at the higher temperature.
3. At both temperatures in salinities of less than 100% sea water, both species were hyperosmotic, but *Ligia pallasii* maintained a relatively higher internal osmotic pressure than did *L. occidentalis*. This is evidenced in 50% sea water at 12° C where the freezing point depression of the haemolymph ($\triangle i$) of *L. pallasii* was 1.67 compared with 1.31 for *L. occidentalis*.
4. At both temperatures in 50% and 25% sea water, survival of *Ligia pallasii* was greater than that of *L. occidentalis*.
5. In neither species was the osmotic concentration of the haemolymph influenced by the size or sex of the animals.
6. When both species were adapted to 150% sea water and then transferred to 50% sea water, animals at 12° C came to a steady internal osmotic pressure later and gained weight more slowly than did the animals at 22° C. In these transferral experiments the per cent body weight gain at both temperatures for *Ligia pallasii* was 5% for the larger males and 8–9% for the smaller females; for *L. occidentalis* it was 9–10% for males and females.
7. The osmoregulatory responses shown by both species correlate well with their overall behavior and ecology. The slower moving *Ligia pallasii* live permanently in cool, moist habitats characterizad by fluctuating, hyposaline conditions. The faster moving *L. occidentalis* alternate their activity between periods of foraging in drier, more terrestrial situations and periods of exposure to wet conditions of variable salinity where they can replace water lost by evaporation.

LITERATURE CITED

BARNES, T. C., 1932. Salt requirements and space orientation of the littoral isopod *Ligia* in Bermuda. *Biol. Bull.*, **63**: 496–504.

BATEMAN, J. B., 1933. Osmotic and ionic regulation in the shore crab, *Carcinus maenas*, with notes on the blood concentration of *Gammarus locusta* and *Ligia oceanica*. *J. Exp. Biol.*, **10**: 355–371.

CROGHAN, P. C., 1958. The mechanism of osmotic regulation in *Artemia salina* (L.): The physiology of the branchiae. *J. Exp. Biol.*, **35**: 234–242.

Dixon, W. J., and F. J. Massey, 1969. *Introduction to Statistical Analysis.* McGraw-Hill Co., New York, 638 pp.

Edney, E. B., 1952. The body temperature of arthropods. *Nature,* **170**: 586–587.

Edney, E. B., 1953. The temperature of woodlice in the sun. *J. Exp. Biol.,* **30**: 331–349.

Edney, E. B., and J. O. Spencer, 1955. Cutaneous respiration in woodlice. *J. Exp. Biol.,* **32**: 256–269.

Giese, A. C., 1962. *Laboratory Manual in Cell Physiology.* The Boxwood Press, Pittsburgh, 188 pp.

Gross, W. J., 1954. Osmotic responses in the sipunculid *Dendrostomum zostericolum. J. Exp. Biol.,* **31**: 402–423.

Kinne, O., 1952. Zur Biologie und Physiologie von *Gammarus duebeni. Kiel. Meeresforsch.,* **9**: 134–150.

Kinne, O., 1953. Zur Biologie und Physiologie von *Gammarus duebeni. Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere,* **64**: 183–206.

Lockwood, A. P. M., 1959. The osmotic and ionic regulation of *Asellus aquaticus* (L.) *J. Exp. Biol.,* **36**: 546–555.

Lockwood, A. P. M., 1960. Some effects of temperature and concentration of the medium on the ionic regulation of the isopod *Asellus aquaticus* (L.). *J. Exp. Biol.,* **37**: 614–630.

Lockwood, A. P. M., 1962 The osmoregulation of Crustacea. *Biol. Rev.,* **37**: 257–305.

Miller, M. A., 1938. Comparative ecological studies on the terrestrial isopod Crustacea of San Francisco Bay region. *Univ. Calif. Publ. Zool.,* **41**: 113–142.

Nemenz, H., 1960. On osmotic regulation of the larvae of *Ephydra cinerea. J. Insect. Physiol.,* **4**: 38–44.

Panikkar, N. K., 1940. Influence of temperature on osmotic behavior of some crustacea and its bearing on problems of animal distribution. *Nature,* **146**: 366–367.

Parry, G., 1953. Osmotic and ionic regulation in the isopod crustacean *Ligia oceanica. J. Exp. Biol.,* **30**: 567–574.

Potts, W. T. W., and G. Parry, 1964. *Osmotic and Ionic Regulation in Animals.* Pergamon Press, Oxford, 423 pp.

Ramsay, J. A., 1949. A new method of freezing-point determination for small quantities. *J. Exp. Biol.,* **26**: 57–64.

Riegel, J. A., 1959a. Some aspects of osmoregulation in two species of sphaeromid isopod Crustacea. *Biol. Bull.,* **116**: 272–284.

Riegel, J. A., 1959b. A revision in the sphaeromid genus *Gnorimosphaeroma* Menzies (Crustacea: Isopods) on the basis of morphological, physiological and ecological studies on two of its "subspecies." *Biol. Bull.,* **117**: 154–162.

Robertson, J. D., 1960. Osmotic and ionic regulation, pp. 317–339. *In:* T. H. Waterman, Ed., *The Physiology of Crustacea.* Academic Press, New York and London.

Todd, M. E., 1963. Osmoregulation in *Ligia oceanica* and *Idotea granulosa. J. Exp. Biol.,* **40**: 381–392.

Vol. 138, No. 2 April, 1970

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

HYDROID AND YOUNG MEDUSA STAGES OF *DIPURENA STRANGULATA* (HYDROZOA, CORYNIDAE)[1, 2]

DALE R. CALDER

Virginia Institute of Marine Science, Gloucester Point, Virginia 23062

The genus *Dipurena* was established by McCrady (1857) for two species of Anthomedusae from Charleston Harbor, South Carolina. Mayer (1910a) synonymized McCrady's genus with *Slabberia* of Forbes (1846), but later (Mayer, 1910b) recognized *Dipurena* as the valid name of the genus because *Slabberia* had been applied to a leptomedusan by Oken (1815).

Kramp (1961) discussed seven species of *Dipurena* but considered at least two of them doubtful, *D. brownei* (Bigelow) and *D. dolichogaster* Haeckel. The validity of *D. pyramis* (Haeckel), a somewhat aberrant species of uncertain systematic position, has also been questioned (Kramp, 1959). The remaining four, *D. halterata* (Forbes), *D. strangulata* McCrady, *D. ophiogaster* Haeckel and *D. reesi* Vannucci, are relatively well-known and recognized species. An additional species, *D. simulans,* was briefly described by Bouillon (1965). Of the latter five, both hydroid and medusa phases are known for all but *D. strangulata,* the type species of the genus and a rather common hydromedusa along the United States east coast. During a recent survey of the Hydrozoa in southern Chesapeake Bay, unidentified capitate hydroids were found on sponges in the York River, Virginia. Through life-history studies the hydroids were linked to the medusa *D. strangulata.* A description of the hydroid and young medusa is the purpose of this paper.

MATERIALS AND METHODS

The hydroid *Dipurena strangulata* was found on colonies of the sponge *Microciona prolifera* collected from oyster grounds by dredging and from pier

---

[1] Contribution No. 338 from the Virginia Institute of Marine Science, Gloucester Point, Virginia 23062.

[2] This paper was written during tenure of a National Research Council of Canada Postdoctoral Fellowship at the Museum of Natural Sciences, National Museums of Canada, Ottawa 4, Ontario.

Copyright © 1970, by the Marine Biological Laboratory
Library of Congress Card No. A38-518

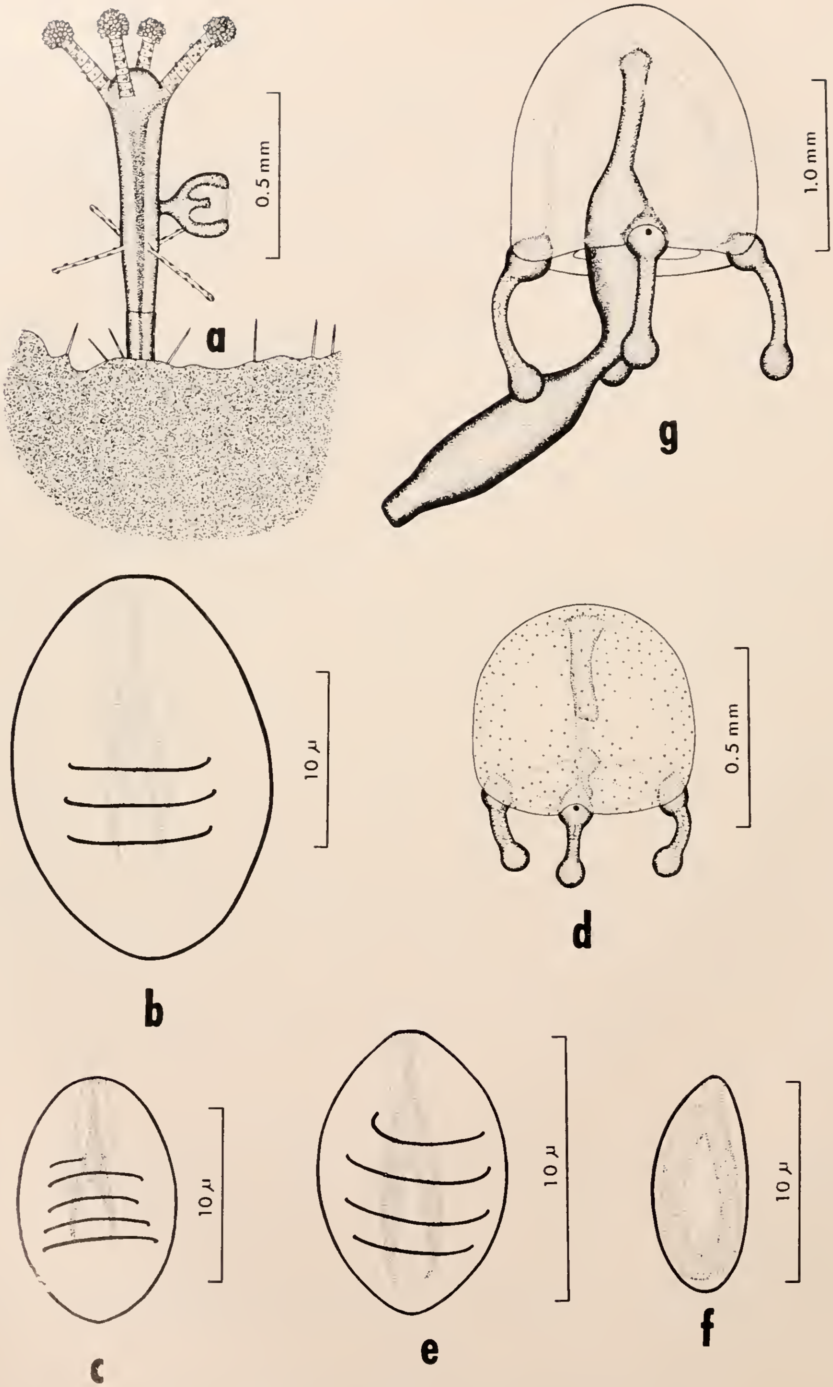


FIGURE 1. *Dipurena strangulata*, a. hydroid, b. large stenotele of hydroid, c. small stenotele of hydroid, d. young medusa, e. stenotele of young medusa, f. desmoneme of young medusa, g. mature, 6-day old laboratory-reared medusa.

pilings by SCUBA diving. All descriptions were made from freshly collected, living specimens, obtained at Gloucester Point, Virginia (37°14.7′N, 76°30.0′W). Medusae were obtained by isolating hydroids with medusa buds in large finger bowls. Liberated medusae were removed to petri dishes containing filtered seawater of 19‰ salinity. Cultures were maintained in an air-conditioned room at 23 ± 3 C, seawater was changed daily, and medusae were fed *Artemia* nauplii. Specific identification was made from medusae reared to the adult stage. The plankton of the York River at Gloucester Point was monitored for medusae at weekly intervals from September 1966 through December 1967 using a #20 mesh plankton net with an opening diameter of 11.5 cm.

DESCRIPTION

Hydroid (Fig. 1a)—Zooids of one type, arising singly from a stolon network embedded in the sponge substrate, perisarc thin, smooth, terminating slightly below the filiform tentacles. Hydranth short, nearly cylindrical when extended, reaching 0.8–1.0 mm above the substrate, 0.15–0.20 mm wide at the distal end, 4 filiform tentacles in a proximal whorl, 0.15–0.19 mm long, 0.03 mm wide, occurring about $\frac{1}{3}$ of the distance apically, 4–6 capitate tentacles in a distal whorl, usually extending above the dome-shaped hypostome, 0.2 mm long, 0.05 mm wide, with 8–11 endodermal cells, terminal knobs 0.08–0.10 mm wide.

Nematocysts (Figs. 1b, c):
stenoteles
large .... 20.6–25.0 × 14.4–18.2 μ (undischarged)
small .... 11.9–17.2 × 7.9–11.3 μ (undischarged)

Medusa (Fig. 1d)—Medusa buds attached by a short stalk to the hydranth just distal to the filiform tentacles, or on blastostyles, a maximum of 2 medusa buds observed concurrently on a single hydranth. Marginal tentacles 4, well developed before liberation, tentacle bulbs 4, of moderate size, each with a single, dark red, abaxial ocellus. Velum broad, mesoglea thin, radial canals and ring canal present, narrow. Manubrium tubular, tapering slightly from a somewhat bulbous base. Exumbrella with a few scattered nematocysts.

At liberation, medusa bell-shaped, 0.55–0.60 mm high, 0.50–0.55 mm wide, manubrium about 0.25 mm long. Each marginal tentacle with a single terminal knob.

Nematocysts (Figs. 1e, f):
stenoteles .... 10.4–11.2 × 6.9–7.8 μ (undischarged)
desmonemes .... 10.0–11.7 × 4.2–4.9 μ (undischarged)

Hydroids of *Dipurena strangulata* were first noticed on 18 June, 1967 at Gloucester Point. The hydroids were relatively common on *Microciona prolifera* and had probably been overlooked previously because of their small size. Medusa buds on these specimens suggested that the hydroid had been active for some time. Specimens were collected regularly at Gloucester Point in depths from 1.5 to 4 m throughout the rest of the summer and autumn until mid-November. During this time water temperatures ranged from a high of 28 C to a low of 10 C and salinities varied approximately from 18‰ to 24‰.

The medusa of *D. strangulata* was first collected at Gloucester Point on 29 June, 1967, and during the summer it was one of the most abundant medusae in the plankton samples. The manubrium of living specimens from the plankton was greenish and the tentacle bulbs varied from pink to red. In laboratory-reared specimens the manubrium turned green one or two days after liberation while the tentacle bulbs were cream-colored. Ocelli were dark red in newly liberated medusae but in older specimens appeared almost black. Gonads developed in cultured medusae after about three days, a constriction divided the gonad into two rings by four days, and by six days the manubrium extended beyond the velum (Fig. 1g). All laboratory-reared medusae still living 12 days after liberation from the hydroid were preserved.

The following specimens of *D. strangulata* have been deposited in the Museum of Natural Sciences, National Museums of Canada:

1. *Dipurena strangulata*, hydratype on *Microciona prolifera,* N.M.C. Cat. No. 568. The term hydratype was introduced by Rees and Thursfield (1965) for material on which an original description is based of a hydroid whose medusa is previously described.
2. *Dipurena strangulata*, young medusa, 10 specimens, N.M.C. Cat. No. 609.
3. *Dipurena strangulata*, adult medusae, 3 specimens. N.M.C. Cat. No. 610.

Discussion

The genus *Dipurena*, belonging to the family Corynidae, is characterized by having the gonads divided into two or more distinct rings about the manubrium of the medusa, and by a *Coryne*-like hydroid, having all tentacles capitate, or with both capitate and reduced filiform tentacles (Russell, 1953). Of the described *Dipurena* hydroids, *D. strangulata* most resembles *D. reesi*. The two species differ markedly from others of the genus in having a single whorl of capitate tentacles. In lacking additional scattered capitate tentacles the hydroids of *D. reesi* and *D. strangulata* resemble *Cladonema radiatum* Dujardin closely. Finding it practically impossible to distinguish *D. reesi* and *C. radiatum* from the descriptions alone, Brinckmann and Petersen (1960) studied the hydroids of both species and discovered that differences existed in: (1) the shape of the knob on the capitate tentacles; (2) the number of endodermal cells in these tentacles; (3) the morphology and complement of nematocysts; and (4) the position and shape of the filiform tentacles. In having stenoteles only, rather than stenoteles and microbasic euryteles, and in having no distinct terminal swelling at the distal end of the filiform tentacles, *D. strangulata,* like *D. reesi*, differs from *C. radiatum*. In certain other characteristics, however, *D. strangulata* differs from *D. reesi* and is similar to *C. radiatum*: (1) having the filiform tentacles about one-third of the distance apically on the hydranth rather than half way; (2) having fewer endodermal cells in the capitate tentacles (7–8 in *C. radiatum*, 8–11 in *D. strangulata,* and about 18 in *D. reesi*).

Although the hydroids of *C. radiatum*, *D. reesi* and *D. strangulata* may be similar, the morphological differences between their medusae are such that the more highly evolved and specialized *C. radiatum* is placed in a separate family (Rees, 1957). Rees noted a greater diversity of form occurs generally in medusae because of the free planktonic phase, while the hydroids, being sedentary, frequently persist in a somewhat simpler form.

A thorough description of the adult medusa of *D. strangulata* was given by Mayer (1910a) but his description of the juvenile does not agree with my observations. In Mayer's specimens the bell was cylindrical with vertical sides, a slight apical projection was present, and two of the four marginal tentacles were undeveloped and represented by basal bulbs. Apparently laboratory culture of these medusae was not undertaken by Mayer and a misidentification could have been made in piecing together the life history from stages in plankton samples. Mayer's description closely resembles the young medusa of *Linvillea agassizi* (McCrady) obtained from the hydroid at Gloucester Point. According to Mayer both of these species were very abundant in Charleston Harbor during summer and early autumn, and he may have mistaken juvenile *L. agassizi* for *D. strangulata*. None of the species of *Dipurena* are known to resemble *Linvillea* in having two of the four marginal tentacles rudimentary at liberation.

While it may occur on other substrates, the hydroid of *D. strangulata* was found only in association with the sponge *Microciona prolifera*. Two other species of the genus were also originally described from a sponge substrate: Rees (1939) described *D. halterata* on the sponge *Haliclona cancellata,* and Bouillon (1965) found *D. simulans* developing in the oscula of the sponge *Adocia simulans*. Vannucci (1956) observed *D. reesi* growing on glass and *Ulva* in an aquarium, and *D. ophiogaster* was found on the stipe of an alga (Rees, 1941).

In addition to *D. strangulata,* several species of *Dipurena* have been described along the United States east coast. *D. cervicata*, described by McCrady (1857) from a single specimen, was synonymized with *D. strangulata* by Mayer (1910a) after a detailed study of the medusae in Charleston Harbor. *D. conica,* described by A. Agassiz in L. Agassiz (1862) from Buzzards Bay, Massachusetts, was also synonymized by Mayer (1910a) with *D. strangulata*. Earlier separation of these two had been based primarily on bell shape and manubrium length, characters showing wide variation in Mayer's specimens of *D. strangulata* from Charleston. Two species described by Mayer (1900) from Tortugas, Florida, *D. picta* and *D. fragilis,* have since been placed in synonymy. *D. picta* was synonymized by Mayer (1910a) with *D. catenata,* a synonym of *D. halterata* (Kramp, 1961). *D. fragilis* was relegated to a subspecies of *D. strangulata* by Mayer (1910a), but Kramp (1961) considered it fully synonymous. Hargitt's (1904) report of *Dipurella clavata* was synonymized by Mayer (1910a) with *D. strangulata*. However, Hargitt's (1902) original description of *D. clavata* appears to have been based on a juvenile specimen of *Linvillea agassizi*. Thus, the genus *Dipurena* is represented by two recognized species along the eastern United States; *D. strangulata* occurring from New England to Florida, and *D. halterata* occurring in Florida.

SUMMARY

The hydroid stage in the life history of *Dipurena strangulata,* a relatively well-known medusa along the United States east coast, is described for the first time. A previous description of the young medusa does not accord with observations recorded here, but is similar to and may have been based on young *Linvillea agassizi* rather than *D. strangulata*. Of the described hydroids of *Dipurena*, *D. strangulata* is most like *D. reesi* morphologically, but differs in the position of the filiform tentacles and the number of endodermal cells in the capitate tentacles. The status of the various species of *Dipurena* described from the North American Atlantic coast is reviewed.

LITERATURE CITED

Agassiz, L., 1862. *Contributions to the Natural History of the United States of America. Volume 4.* Little, Brown and Co., Boston, 380 pp.

Bouillon, J., 1965. Diagnoses préliminaires de trois hydroides de Roscoff, p. 54. *In:* G. Teissier, *Inventaire de la Faune Marine de Roscoff, Cnidaires et Cténaires.* [deuxieme édition]. Edit. Sta. Biol. Roscoff.

Brinckmann, A., and K. W. Petersen, 1960. On some distinguishing characters of *Dipurena reesi* Vannucci, 1956 and *Cladonema radiatum* Dujardin, 1843. *Pubbl. Sta. Zool. Napoli,* **31**: 386–392.

Forbes, E., 1846. On the pulmograde medusae of the British seas. *Ann. Mag. Natur. Hist., Series* 1, **18**: 284–287.

Hargitt, C. W., 1902. Notes on a few medusae new to Woods Holl. (sic) *Biol. Bull.,* **4**: 13–23.

Hargitt, C. W., 1904. The medusae of the Woods Hole region, Massachusetts. *Bull. Bur. Fish.,* **24**: 21–79.

Kramp, P. L., 1959. The Hydromedusae of the Atlantic Ocean and adjacent waters. *Dana Rep., Carlsberg Found.,* **46**: 1–283.

Kramp, P. L., 1961. Synopsis of the medusae of the world. *J. Mar. Biol. Ass. U. K.,* **40**: 1–469.

Mayer, A. G., 1900. Some medusae from the Tortugas, Florida. *Bull. Mus. Comp. Zool. Harvard Univ.,* **37**: 13–82.

Mayer, A. G., 1910a. Medusae of the world. Volume I. The Hydromedusae. *Carnegie Inst. Washington Publ.,* **109**: 1–230.

Mayer, A. G., 1910b. Medusae of the world. Volume III. The Scyphomedusae. *Carnegie Inst. Washington Publ.,* **109**: 499–735.

McCrady, J., 1857. Gymnopthalmata of Charleston Harbor. *Proc. Elliott Soc. Natur. Hist. Charleston,* **1**: 103–221.

Oken, L., 1815. *Lehrbuch der Naturgeschichte.* Teil 3, Zool., Jena.

Rees, W. J., 1939. The hydroid of the medusa *Dipurena halterata* (Forbes). *J. Mar. Biol. Ass. U. K.,* **23**: 343–346.

Rees, W. J., 1941. Notes on British and Norwegian hydroids and medusae. *J. Mar. Biol. Ass. U. K.,* **25**: 129–141.

Rees, W. J., 1957. Evolutionary trends in the classification of capitate hydroids and medusae. *Bull. Brit. Mus. (Natur. Hist.) Zool.,* **4**: 455–534.

Rees, W. J., and S. Thursfield, 1965. The hydroid collections of James Ritchie. *Proc. Roy. Soc. Edinburgh,* **69B**: 34–220.

Russell, F. S., 1953. *The Medusae of the British Isles.* Cambridge University Press, Cambridge, 530 pp.

Vannucci, M., 1956. Biological notes and description of a new species of *Dipurena* (Hydrozoa, Corynidae). *Proc. Zool. Soc. London,* **127**: 479–487.

Reference: *Biol. Bull.*, **138**: 115–128. (April, 1970)

DISTRIBUTION AND METABOLISM OF α-ECDYSONE IN PUPAE OF THE SILKWORM *ANTHERAEA POLYPHEMUS*[1]

LUCY CHERBAS AND PETER CHERBAS

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

The insect steroid α-ecdysone is known to provoke the adult development of diapausing insect pupae or even of isolated segments of pupae (Williams, 1970). Within 12–24 hours after the injection of 5–10 μg of the hormone into a diapausing saturniid pupa, the animal's respiration increases dramatically, and striking increases in the rates of synthesis of DNA, RNA, and protein can be demonstrated (Wyatt, 1968). These metabolic events precede by 3 or 4 days the first gross morphological change, the retraction of the epidermis from the overlying pupal cuticle.

Attempts to understand ecdysone's action at the molecular level are complicated by striking gaps in present knowledge. For example, ecdysone's target tissues have not yet been identified. Nor do we know whether it is unaltered α-ecdysone or a derivative which reacts with these tissues. The object of the present investigation was to clarify some of these matters by studying the fate of injected α-ecdysone, in particular, its chemical transformations and interactions with the tissues. Recent studies of estradiol in mammalian systems (Jensen and Jacobson, 1962; Gorski, Toft, Shyamala, Smith and Notides, 1968) have demonstrated the potential power of this approach. Some experiments of this kind on larval *Calliphora* have been reported by Karlson, Sekeris and Mauer (1964).

In the present communication we describe the distribution and metabolism of α-ecdysone in polyphemus pupae. Future publications will discuss the binding of the hormone in greater detail.

Materials and Methods

Experimental animals

Diapausing pupae of the silkworm, *Antheraea polyphemus*, were purchased from dealers and stored at 5° C until immediately before use. The pupae used in these experiments were chilled 5–10 months; though some began visible development within a day after removal from the cold, none exhibited retraction of the epidermis at the beginning of any experiment. Male pupae were used exclusively, since their large antennae were a convenience in some experiments.

Reagents

β-ecdysone was purchased from Mann Research. Other ecdysones were supplied as follows: synthetic α-ecdysone, synthetic α-ecdysone-23,24-^{3}H (7.3

---

[1] This study was supported, in part, by an NSF predoctoral fellowship to Lucy Cherbas and by an NSF grant to Professor C. M. Williams.

c/mmole), 25-deoxyecdysone-^{3}H, by Dr. John Siddall of Zoecon Corporation; ponasterone A, ponasterone C, by Professor K. Nakanishi of Columbia University; rubrosterone, by Professor T. Takemoto of Tohoku University. The radiochemical purity of the α-ecdysone-^{3}H was greater than 99% as determined by thin layer chromatography. Inulin-^{3}H (200 mc/g) was purchased from Schwarz BioResearch; water-^{3}H, from New England Nuclear Corporation.

Injections

Injections were made through the thoracic tergum using a 26-gauge hypodermic needle on a plastic 1 ml syringe controlled by an Agla micrometer. Each syringe was calibrated before use. Injections were routinely made without anesthesia, the puncture wound being promptly sealed with melted wax. Ecdysones were dissolved in 10% 2-propanol and injected in volumes not exceeding 50 μl; inulin-^{3}H and water-^{3}H were injected in no more than 50 μl of water.

Blood samples

Blood samples, except where otherwise indicated, were squeezed from a small incision in the integument at the edge of an antenna. When successive samples were taken from the same pupa, the incision was opened for each sample, and then resealed with melted wax.

Washing of antennae

Each antennae was removed, intact, separated from the overlying cuticle, and placed immediately into a beaker containing 50 ml of pupal saline (Weevers, 1966, modified by the substitution of sucrose for glucose and by the addition of 0.01 *M* thiourea and 0.1% penicillin) at room temperature. By means of a 23-gauge hypodermic needle, about 7 ml of this solution was injected through the main longitudinal blood space of the antenna; the injection rate was just sufficient to keep the antenna distended. The beaker was then placed on a low-speed gyratory shaker for 15 min, the solution being changed twice.

Extraction of α-ecdysone and its metabolites

Two extraction procedures were used: (A.) 0.2–0.5 ml of frozen blood was thawed and added to 2 ml of cold methanol. The resulting suspension was centrifuged at 12,000 × g for 10 min. The pellet was washed with 1 ml of cold methanol and again centrifuged. The supernatants were combined and the solvent evaporated under nitrogen at room temperature to approximately the original volume of the blood; the methanolic extract was stored at −20° C until used. Any precipitate that formed during storage was discarded.

(B.) 10 μl of blood was removed from a pupa and added to 50 μl of methanol at room temperature. (Occasionally, 25 μl of blood and 100 μl of methanol were used.) The mixture was shaken vigorously, stored at −20° overnight, and then centrifuged. The methanolic supernatant was used for chromatography. This method permitted sequential analyses of the blood of a single pupa.

Thin layer chromatography

Plastic-backed thin layer plates of silica gel containing fluorescent indicator (Eastman "Chromagram") were stored over calcium sulfate as desiccant. 10 μl samples were spotted after the addition of α-ecdysone and β-ecdysone (4 μg each) as markers. The solvent system was that of Horn, Middleton and Wunderlich (1966) (4 parts chloroform, 1 part 96% ethanol). The developed chromatogram was examined under an ultraviolet lamp, and the positions of the marker ecdysones were observed as dark spots. The chromatogram was then cut into strips 1–1½ cm wide, each containing the path of one sample. Each strip was cut into sections 2 mm long in the region containing α- and β-ecdysones, and 5 mm long elsewhere. The radioactivity in each section was measured in a liquid scintillation counter as described below. The pattern of radioactivity was analyzed graphically, using cpm/cm as the ordinate, and the position of the center of the section as abscissa.

Scintillation counting

Radioactivity was measured in a liquid scintillation counter, using 10 ml of a scintillation fluid consisting of 1 part Bio Solv type 3 (Beckman Instruments) to 10 parts toluene counting solution (0.4% 2,5-diphenyloxazole and 0.005% 1,4,bis-2-(methyl-5-phenyloxazolyl)-benzene in toluene). Tissue samples were placed in a scintillation vial containing 0.1 ml of a solution of sodium ethylenediaminetetraacetate (15 mM) and sodium dodecyl sulfate (1%), and warmed about 10 minutes on a hotplate before the counting solution was added. We were able to count whole antennae in this manner without significant quenching. Blood samples and sections of thin layer plates were added directly to the scintillation fluid. The customary 10 μl blood samples gave no quenching; the sections of thin layer plates gave substantial, but reproducible, quenching.

RESULTS

The distribution of injected inulin and water

We studied first the rates at which two model compounds, inulin and water, are distributed after being injected into the hemocoel. We also determined the equilibrium distribution volume of each compound, *i.e.,* the volume through which each compound would have to be distributed to give the observed concentration, if the distribution were even.

Radioactive inulin was injected into a series of pupae *via* the right half of the thoracic tergum, and 10 μl blood samples were collected repeatedly from the left forewing and antenna of each animal. In 50% of the pupae, the inulin concentration reached equilibrium within two hours. Figure 1a shows a typical result. Figure 1b is included to demonstrate that before equilibrium is reached the concentrations at two separate sites sometimes differ dramatically. The inulin volume remained constant (± 4%) for 24 hours after equilibrium was attained.

From the specific activity of the blood samples at equilibrium, we calculated the distribution volume. Figure 2 shows the equilibrium distribution volume for inulin as a function of live weight. The inulin volume is approximately 0.49 ml per

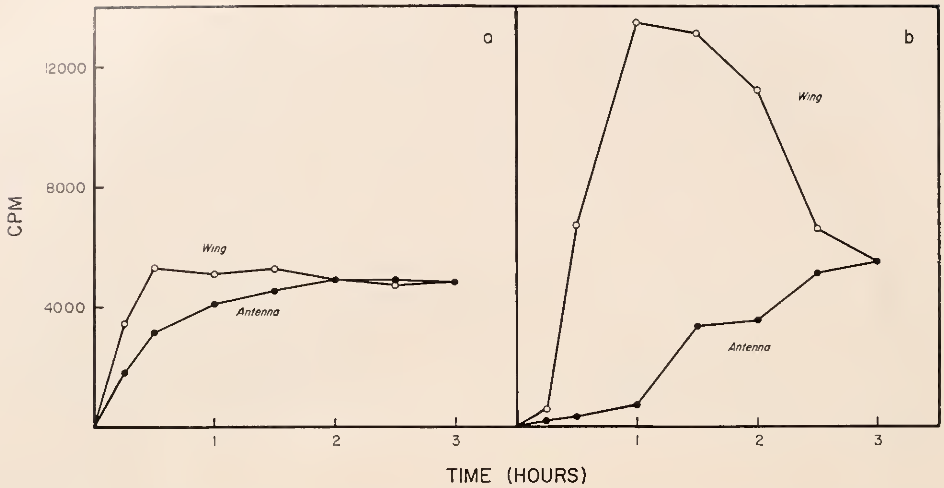


FIGURE 1. Distribution of inulin by the blood. Parts a and b represent different pupae; each was injected with 50 μl of inulin-^{3}H (7×10^5 cpm) via the thoracic tergum. The animals were kept at 25° C and 10 μl blood samples were taken at the times indicated. Abscissa: cpm per 10 μl of blood; open circles: blood samples taken from the wing; closed circles: blood samples taken from the antenna.

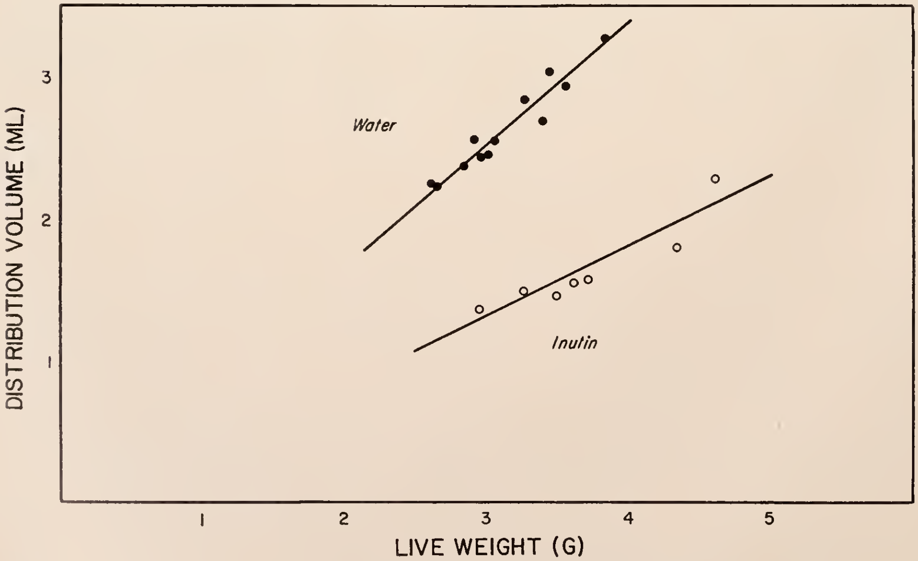


FIGURE 2. Distribution volume of inulin and water as a function of live weight. Each pupa received 50 μl (3×10^5 cpm) of inulin-^{3}H in water or 50 μl of water-^{3}H (6×10^6 cpm) injected *via* the thoracic tergum. After 3 hours (inulin) or 25 hours (water) at 25° C, a 10 μl aliquot of blood was removed from the antennal region and counted. The lines were calculated by the least-squares method.

g live weight. For reasons to be discussed later, we will assume that inulin is confined to the blood.

The water volume was measured in the same way and is approximately 0.86 ml per g live weight (Figure 2). The time required for water-^{3}H to reach its equilibrium concentration was not measurably different from that required for the equilibration of inulin. The water volume remained constant for at least 24 hours after equilibrium was attained.

The distribution of injected ecdysone

Radioactive α-ecdysone, injected in doses ranging from 1.0 to 8.6 μg per pupa, approached its equilibrium concentration in the blood of the antenna at a rate indistinguishable from that for inulin. This situation was not altered when 100 μg of unlabeled β-ecdysone and 1.2 μg of radioactive α-ecdysone were injected together: the radioactive material still reached its equilibrium distribution volume in about two hours.

In another experiment a large number of pupae were injected with 0.1 to 10 μg of α-ecdysone-^{3}H, and the blood of each pupa was sampled once during the 48 hours following the injection. Figure 3 illustrates this experiment for four different doses of ecdysone. Here the ordinate, the fraction of the radioactivity remaining in the blood, is simply the estimated inulin volume divided by the observed ecdysone volume. It is to be emphasized that the term "ecdysone volume" refers in fact to the distribution volume of radioactive α-ecdysone and its *radioactive* metabolites. There is a plateau between 3 and 36 hours, during which time the ecdysone volume is essentially constant and equal to 1.03 ± 0.13 (S. D.) ml per g live weight. This figure implies that 47% of the radioactivity is present in the blood. Only in the case of the 10.4 μg dose, which is sufficient to provoke adult development, are there pronounced deviations from the "equilibrium" level of 47%.

The ecdysone concentration in fat body and Malpighian tubules

Two pupae were injected with 1 μg and 10 μg doses of α-ecdysone-^{3}H (10^7 dpm in each case). One day later the pupae were dissected. Most of the Malpighian tubules were removed from each pupa, rinsed in saline, and tested for radioactivity. The cellular volume of this tissue is negligible; the radioactivity did not exceed background. Two other pupae were injected with 1 μg and 10 μg of α-ecdysone, respectively. Twenty-nine hours later 25–50 μl of fat body, including a small amount of blood, was removed with a pipette and counted. Each sample contained approximately the amount of radioactivity found in an equal volume of blood from the same pupa. Hence we conclude that the ecdysone that disappeared from the blood was not concentrated in the fat body or Malpighian tubules in the form of α-ecdysone or any radioactive metabolite of α-ecdysone.

The ecdysone concentration in the epidermis

Measurement of the radioactivity in an antenna was complicated by the large volume of blood trapped within this hollow organ. In order to measure the radioactivity associated with the cells of an unwashed antenna, we performed the

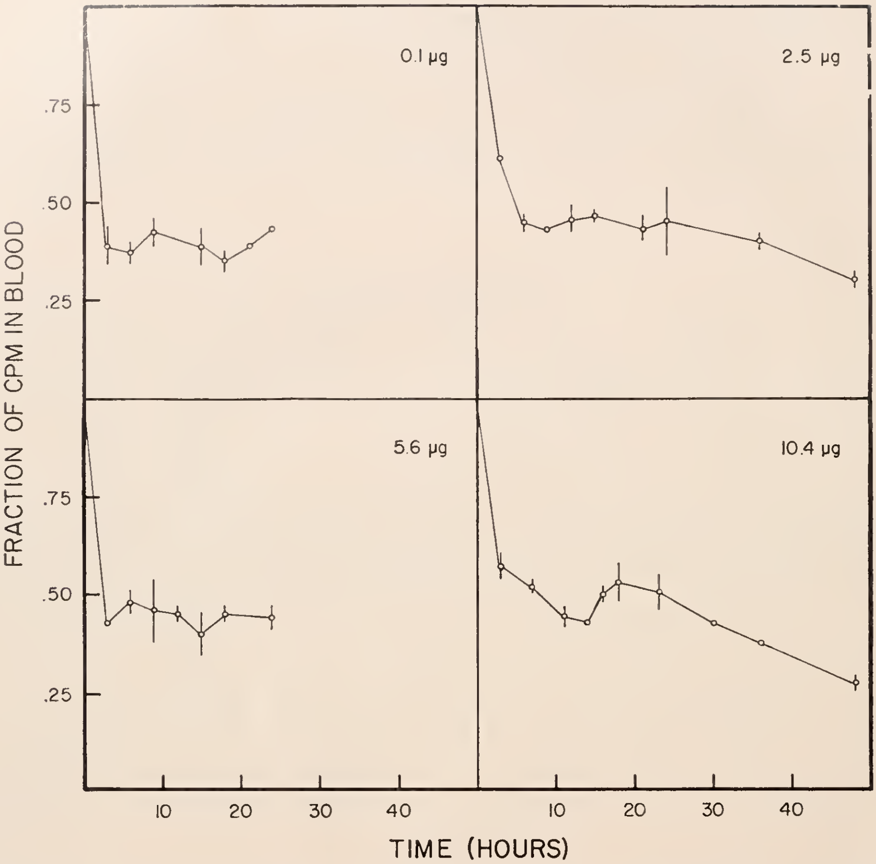


FIGURE 3. Fraction of radioactivity remaining in the blood after an injection of α-ecdysone-^{3}H at time zero. Each point is an average of values from two pupae; the range of individual values is indicated. In calculating the fractions, it was assumed that the blood volume is equal to the inulin volume shown in Figure 2.

following experiment. One series of pupae received inulin-^{3}H, and a second received α-ecdysone-^{3}H (2.5 μg/pupa). The antennae were removed from each pupa seven hours later; a blood sample was collected at the same time. Each antenna was blotted carefully and its volume measured by drawing it into a calibrated glass capillary. Assuming inulin to be present only in the blood, we found that about 70% of the volume of a blotted antenna was blood. This information enabled us to calculate the specific activity of the cells from pupae that had received α-ecdysone. We found no difference between the specific activity of the cells and that of the blood. Only differences greater than 30% would have been detected by this method.

Another group of pupae were injected with 0.1 to 10 μg of α-ecdysone-^{3}H. After 3–49 hours, we removed and washed their antennae as described under Methods, and counted the radioactivity. We had determined, using inulin, that an antenna washed in this manner retains about 0.2 μl of blood. Therefore, blood samples were also counted and the radioactivity in each antenna was corrected for contamination with 0.2 μl of blood. In most cases, each antenna contained an amount of radioactivity equivalent to 1.4 ± 0.7 (S. D.) μl of blood. (Some important exceptions to this result are described in the Discussion.) Despite the uncertainties in the volume measurements, this experiment and the preceding one demonstrate that ecdysone and its radioactive metabolites in antennal tissue are largely unaffected by this washing procedure.

The metabolic products of α-ecdysone

Each of ten pupae was injected with 1.2 μg of α-ecdysone-^{3}H. At intervals from 3 to 24 hours later, pupae were exsanguinated and the blood extracted according to Method A. The final methanol fractions contained 82 (± 4)% of the radioactivity in the blood samples. There was no variation of extraction efficiency with time between injection and extraction. A control blood sample, to which α-ecdysone-^{3}H was added after bleeding, was also extracted with 82% efficiency. As is shown below, α-ecdysone is converted into a variety of metabolites during this 24-hour period; the invariance of the extraction efficiency gave assurance that the extraction procedure did not exclude any significant, radioactively-labeled metabolite of α-ecdysone, *i.e.*, any metabolite containing more than 5% of the radioactivity. More reproducible kinetic data on the appearance of metabolites were obtained using sequential samples from a single pupa, extracted according to Method B. Here again, no systematic variation in the efficiency of extraction was noted.

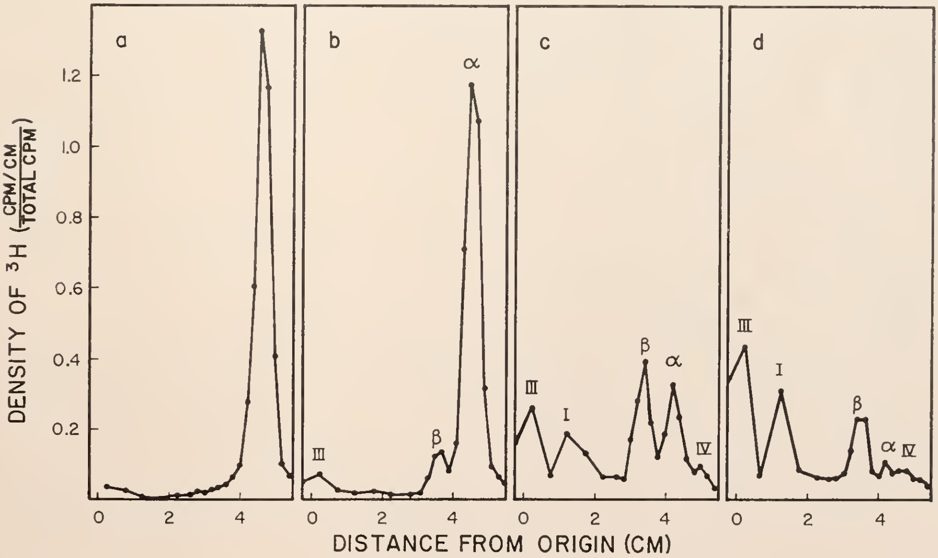


FIGURE 4. Patterns of radioactivity in thin-layer chromatograms of blood extracts, after injection of α-ecdysone-^{3}H. (a) Control, to which the α-ecdysone-^{3}H was added immediately before extraction. (b), (c), (d) Blood from a single pupa, taken 2 hr, 7 hr, and 11 hr, respectively, after injection of 1.2 μg of α-ecdysone-^{3}H. Solvent front was at 18 cm; there was no significant radioactivity in the portion of the chromatograms not shown.

Thin layer chromatography of the extracts distinguished five metabolites of α-ecdysone. Figure 4 shows the dramatic changes in the pattern of metabolites with time, as seen in a pupa injected with 1.2 μg of α-ecdysone-^{3}H. The metabolite labeled β migrates with pure β-ecdysone in this solvent system as well as in cyclohexane-butanol (6:4). The other metabolites resolved in these chromatograms are designated I, III, and IV. Metabolite IV is found in trace quantities in most extracts, but is not seen in control extracts such as that in Figure 4a. Metabolite I does not appear after injection of 8.6 μg of α-ecdysone; instead, another metabolite of slightly different mobility, labeled II, is seen. The mobilities of all these metabolites, as well as those of a number of ecdysone analogs, are shown in Table I.

The quantity of each component was determined from graphs such as those in Figure 4. The minor component IV never represented a fraction high enough to be estimated accurately. Figure 5 shows the time course of appearance of the various metabolites after injection of 1.2 or 8.6 μg of α-ecdysone-^{3}H. There is an initial lag time; all of the α-ecdysone curves extrapolate to 100% at 1–2 hours after

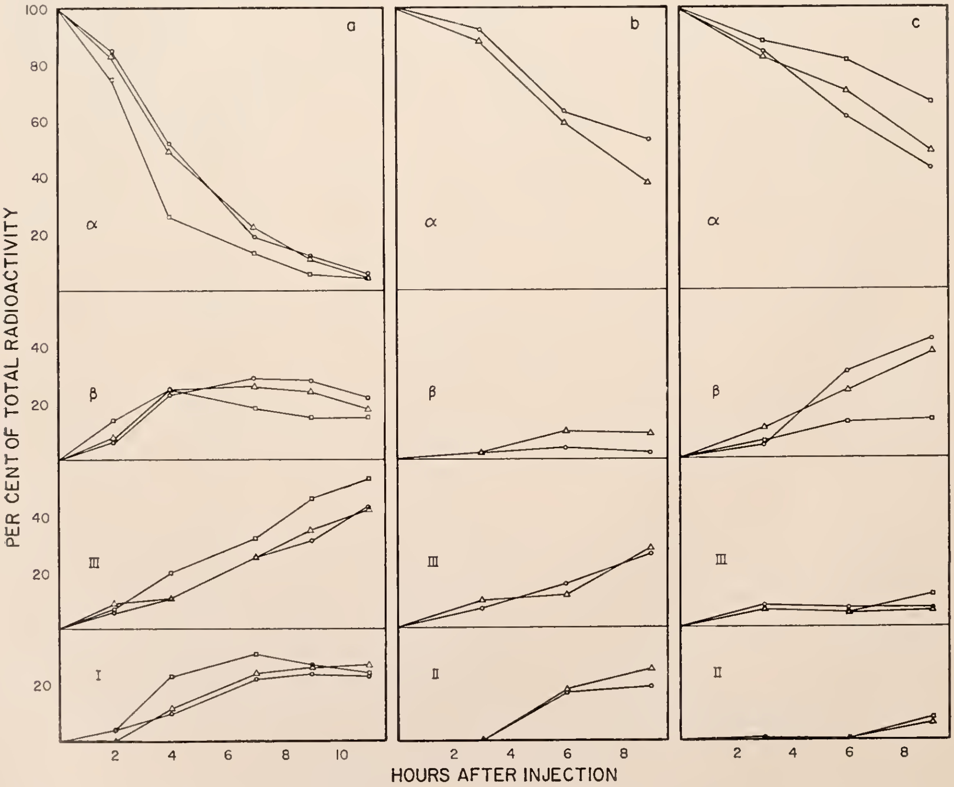


FIGURE 5. Per cent of radioactivity in α-ecdysone and its major metabolites after injection of 1.2 μg of α-ecdysone-^{3}H (a), 8.6 μg of α-ecdysone-^{3}H (b), or 1.2 μg of α-ecdysone-^{3}H and 100 μg of cold β-ecdysone (c) per pupa. II did not appear in series (a); I did not appear in (b) and (c) (see Discussion). In each experiment, the individual pupae are distinguished by different symbols.

TABLE I

Migration in thin-layer chromatography of α-ecdysone, its metabolites, and some ecdysone analogs of known structure

Compound	Structure	$\frac{R_f}{R_f \text{ of } \alpha\text{-ecdysone}}$ (range in parentheses)
α-ecdysone	5β-cholest-7-en-6-one, 2β, 3β, 14α, 22, 25-pentahydroxy (22R) (Huber and Hoppe, 1965)	1.00
β-ecdysone	5β-cholest-7-en-6-one, 2β, 3β, 14α, 20, 22, 25-hexahydroxy (20R, 22R) (Hoffmeister, Gruetzmacher, and Duennebeil, 1967)	0.76 (0.69–0.84)
Metabolite I		0.30 (0.26–0.36)
Metabolite II		0.45 (0.41–0.50)
Metabolite III		0.05 (0–0.06)
Metabolite IV		1.18 (1.04–1.24)
25-deoxyecdysone	5β-cholest-7-en-6-one, 2β, 3β, 14α, 22-tetrahydroxy (22R)	1.50
Rubrosterone	5β-androst-7-en-6, 17-dione, 2β, 3β, 14α-triol (Takemoto, Hikino, Hikino, Ogawa, and Nishimoto, 1968)	1.30
Ponasterone A	5β-cholest-7-en-6-one, 2β, 3β, 14α, 20, 22-pentahydroxy (20R, 22R) (Huppi and Siddall, 1968)	1.45
Ponasterone C	5β-cholest-7-en-6-one, 2α, 3α, 14α, 20, 22, 24-pentahydroxy (Nakanishi, Koreeda, Chang, and Hsu, 1968)	1.35

injection, the time required for distribution of the blood. Excluding the lag time, the decay of α-ecdysone after injection of 1.2 μg is exponential; it is more nearly linear after injection of 8.6 μg. Figure 6 shows the dependence of the rate of breakdown of α-ecdysone upon the concentration of injected α-ecdysone.

When 1.2 μg of α-ecdysone-^{3}H and 100 μg of cold β-ecdysone were injected simultaneously, radioactivity accumulated in β-ecdysone, while II and III were scarcely detectable (Fig. 5c). The loss of counts from α-ecdysone was markedly slower than when the same dose of α-ecdysone was injected alone.

DISCUSSION

Inulin as a label for blood

There is abundant evidence that inulin is excluded from vertebrate cells (Davson, 1964). There are several reasons to believe that this is also true of cells in polyphemus. The inulin volume is the same as the pupal blood volume, estimated by exsanguination. The distribution volume is constant for at least 24 hours; hence inulin is not slowly absorbed or converted to a form that can penetrate cells. After injection of inulin-^{3}H, careful washing of the epidermis removes nearly all radioactivity. The confinement of inulin to the extracellular space makes this compound a very useful tool, both for following the movement of blood and for measuring blood contamination of tissue samples. Levenbook (1958) has already reported the use of ^{14}C-carboxy-inulin for the latter function.

The rates of distribution of inulin and water

It is no surprise that the blood of insects mixes slowly. Craig and Olson (1951) reported, for example, that 20–30 minutes are required for complete mixing to occur in the squashbug *Anasa tristis* and the cabbage bug *Murgantia histrionica.* It is not unreasonable, then, that two hours should be required in the much larger, diapausing polyphemus pupa. What was surprising was the capricious quality of the insect circulatory system as illustrated by the diverse patterns of mixing in Figure 1: at one point in Figure 1b, there is a fourteen-fold difference in inulin concentration between wing and antenna. The path of injected material, and presumably of an endogenous secretion as well, is not predictable. If hormone binding and breakdown are spatially separated, even a very high binding constant might not ensure efficient utilization of a hormone.

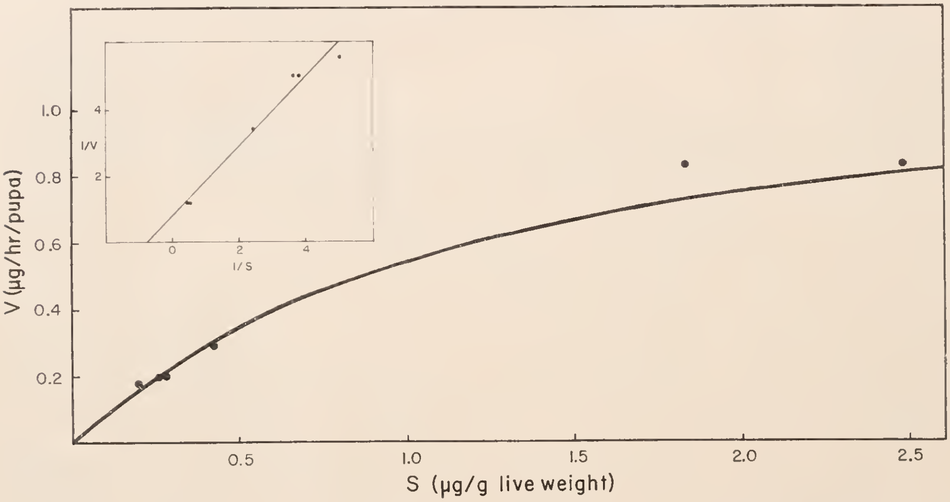


FIGURE 6. The initial rate of metabolism of α-ecdysone as a function of injected dose. The rate was calculated from the first two experimental values in graphs such as those in Figure 5; thus, the lag time was excluded from the calculation. The abscissa (S) is the injected dose divided by the pupa's weight. The curve is plotted from the least-squares best line in the reciprocal plot in the inset.

The rate of distribution of water was not measurably different from that of inulin. From this observation, the *minimum* rate of exchange of water between blood and cells can be calculated to be three-quarters of the amount of water in the blood per hour.

The distribution of ecdysone

Ecdysone reaches its "equilibrium" concentration within about 2 hours after injection. If a carrier is involved, either in the blood or in the target cells, it should be possible to saturate it, making it the rate-limiting step in the hormone's equilibration. The fact that doses up to 10 μg of α-ecdysone or 100 μg of β-ecdysone did not reduce the rate of distribution suggests that a carrier is not involved. In preliminary experiments, we have attempted to detect macromolecules in the plasma capable of binding α-ecdysone. These experiments, using the gel-filtration technique of Hummel and Dreyer (1962), have shown that if carrier macromolecules are present in the plasma of diapausing pupae, they are incapable of binding more than 10% of the α-ecdysone even when the latter is present at the extremely low concentration of 2×10^{-4} μg/ml.

At "equilibrium," about half of the injected ecdysone has disappeared from the blood. The precise value varies slightly with dose and time, but for a given dose of ecdysone, the level of radioactivity in the blood is fairly constant for at least 36 hours. The fact that the amount of radioactivity in the tissues does not change markedly as the ecdysone is metabolized implies that the bulk of the material in both cells and blood is in solution rather than bound to a receptor. Since the ecdysone volume is 20% larger than the water volume, either 20% of the ecdysone is bound or dissolved in a non-aqueous phase, or ecdysone and its metabolites are slightly concentrated by an active transport mechanism; we cannot distinguish among these possibilities.

The experiments with Malpighian tubules and fat body, as well as the detailed experiments with antennae, gave results consistent with the idea that ecdysone is distributed evenly through the tissues and not accumulated by any one tissue. If all tissues are like the antenna in this respect, they contain ecdysone at approximately its concentration in the blood, and the hormone cannot be removed by a washing procedure which removes essentially all the contaminating blood. The fraction of the injected ecdysone associated with tissues in this way does not increase even when the injected dose is only 0.01 molting unit.

Preliminary experiments indicate that the situation just described prevails in antennal tissue during the 48 hours following injection. Superimposed on this baseline are short periods during which the tissue contains up to 20 times as much radioactivity. One such period occurs 5–6 hours after the injection, and another occurs about 18 hours later. If one extracts antennae containing the baseline amount of radioactivity, α-ecdysone and its metabolites are recovered in proportion to their concentrations in the blood. Details of these experiments will be published later.

If all tissues undergo the periods of extensive binding exemplified by the antenna, these periods must be asynchronous, since synchronous binding of such magnitude would remove all of the ecdysone from the blood. Since there is progressive degradation of active hormone, the amount of ecdysone that must be injected to induce adult development must be determined not only by the number of receptors, but also by the necessity to maintain sufficient active hormone during later periods of binding. Detailed analysis of this situation will become possible only when we know whether it is α-ecdysone or one of its metabolites which is active.

Isolated receptor molecules generally exhibit high affinities for hormones; one expects that this will be true of ecdysone receptors. In contrast, the relation between intact tissues and ecdysone can be described formally as weak affinity.

The metabolism of α-ecdysone

King and Siddall (1969) have provided convincing evidence that insects can convert α-ecdysone to β-ecdysone. We have confirmed this observation in polyphemus pupae; the radioactive metabolite labeled β in our chromatograms not only migrated with authentic β-ecdysone in two different solvent systems, but also accumulated when authentic β-ecdysone was injected.

It seems clear that β-ecdysone is the first metabolite formed from α-ecdysone. Not only is this consistent with the structures of the two compounds and with the kinetic data in Figures 5a and 5b, but it is demonstrated in no uncertain terms by the experiment of Figure 5c. Here β-ecdysone accumulates while the production of other metabolites is retarded. Apparently, at least in polyphemus pupae, the enzyme responsible for the conversion of α to β approaches saturation in the range of α-ecdysone concentrations necessary to provoke adult development (Fig. 6). It is also apparent from a comparison of Figures 5a and 5c that β-ecdysone or one of its metabolites inhibits the conversion of α-ecdysone to β-ecdysone, or else β-ecdysone is converted to α-ecdysone at a significant rate.

The chromatographic mobilities of the metabolites are consistent with their being formed by progressive hydroxylation of α-ecdysone. Ohtaki, Milkman and Williams (1968) have shown that oxygen is necessary for the inactivation *in vivo* of α-ecdysone.

We observe two separate metabolites with mobilities 0.3 to 0.5 times that of α-ecdysone. In the presence of large quantities of α- and β-ecdysone we see metabolite II rather than metabolite I (Fig. 5). A variety of hypothetical pathways are compatible with this kinetic data: I as a precursor of II; II as a precursor of I; or both I and II produced from β along separate pathways.

Thompson, Kaplanis, Robbins and Yamamoto (1967) reported the isolation from the tobacco hornworm of two ecdysone-like materials with mobilities less than β-ecdysone in a chromatographic system similar to ours. One compound was 20,26-dihydroxy-α-ecdysone—a biologically active substance which they suggested might be a product of β-ecdysone. Both its structure and its chromatographic properties indicate that it may be I or II.

Metabolite IV differs from the other radioactive metabolites in being somewhat less polar than α-ecdysone. It appears quite early and never amounts to more than 2% of the radioactivity. For the time being, we defer any further consideration of its significance.

Metabolite III is so immobile in our chromatographic system that it is impossible to be certain that it represents only one compound. The progressive accumulation of this metabolite(s) suggests that it may be at the end of a degradative pathway for α-ecdysone. Since the injected α-ecdysone was labeled on carbons 23 and 24 of the side-chain, the possibility should not be overlooked that III is what remains of the side-chain after oxidative cleavage of a previous metabolite. This kind of degradative pathway has already been suggested by Horn *et al.* (1966).

Previous studies using biological assays (Ohtaki *et al.*, 1968; Ohtaki and Williams, 1970; Karlson and Bode, 1969) have demonstrated exponential inactivation of injected α-ecdysone in dipteran larvae and pupae of the silkworm *Samia cynthia*. Until we know which of the radioactive metabolites have biological activity, a detailed comparison of these studies with ours is not possible.

We gratefully acknowledge the advice of Professor C. M. Williams and his critical reading of the manuscript. Radioactive α-ecdysone was essential to this study and we are indebted to Dr. John Siddall for making it available to Professor Williams' laboratory. We are also grateful to Professor Fotis C.

Kafatos, Professor Lynn M. Riddiford, and Mr. Julian Shepherd for reading and criticizing the manuscript.

After the preparation of this paper, an independent study of ecdysone metabolism came to our attention. Professor K. Nakanishi generously allowed us to read a manuscript describing the results of this study (Moriyama, King, Nakanishi, Okauchi, Siddall and Hafferl, 1970). These authors describe four polar metabolites of α-ecdysone. The first to appear is β-ecdysone; the other three, which they call A, B, and C, correspond to our III, I and II, respectively. A less polar peak, D, corresponding to IV, can also be seen in their chromatograms. They discuss possible structures for some of these metabolites. The results of the two studies are entirely consistent, differing only in quantitative details.

SUMMARY

1. The distribution of inulin, water, and α-ecdysone was studied in pupae of the silkworm *Antheraea polyphemus*. All three compounds reach equilibrium within two hours after injection.

2. Inulin is restricted to the extracellular space, which amounts to 0.49 ml per g live weight.

3. The ecdysone space is slightly larger than the water space. About 50% of the radioactivity derived from α-ecdysone remains in the blood for at least 36 hours.

4. The other 50% is associated with cells in a form which cannot be removed by extensive washing of the intact tissues with saline.

5. Transport of ecdysone to the tissues does not involve a rate-limiting carrier system that can be saturated by 10 μg α-ecdysone or 100 μg β-ecdysone.

6. There is no evidence that ecdysone is accumulated by any one tissue or excretory organ.

7. The metabolism of α-ecdysone was studied by thin layer chromatography of extracts of blood after injection of radioactive α-ecdysone. α-ecdysone is rapidly converted to β-ecdysone and the latter to several more polar metabolites.

8. β-ecdysone either inhibits the conversion of α-ecdysone to β-ecdysone or is itself converted to α-ecdysone at a significant rate.

LITERATURE CITED

Craig, R., and N. A. Olson, 1951. Rate of circulation of body fluid. *Science*, **113**: 648–650.

Davson, H., 1964. *A Textbook of General Physiology.* [Third edition] Little, Brown, Boston, 1166 pp.

Gorski, J., D. Toft, G. Shyamala, D. Smith, and A. Notides, 1968. Hormone receptors: studies on the interaction of estrogen with the uterus. *Recent Progr. Hormone Res.*, **24**: 45–80.

Hoffmeister, H., H. F. Gruetzmacher and K. Duennebeil, 1967. Untersuchungen über die Struktur und biochemische Wirkung von Ecdysteron. *Z. Naturforsch.*, **22b**: 66–70.

Horn, D. H. S., E. J. Middleton and J. A. Wunderlich, 1966. Identity of the moulting hormones of insects and crustaceans. *Chemical Communications*, **1966**: 339–341.

Huber, R., and W. Hoppe, 1965. Die Kristall- und Molekülstrukturanalyse des Insektenverpupungshormons Ecdyson mit der automatisierten faltmolekülmethode. *Chem. Ber.*, **98**: 2403–2424.

Hummel, V. P., and W. J. Dreyer, 1962. Measurement of protein-binding phenomena by gel filtration. *Biochim. Biophys. Acta*, **63**: 530–532.

Huppi, G., and J. B. Siddall, 1968. Steroids. CCCXXVI. Synthetic studies on insect hormones. 6. The synthesis of ponasterone A and its stereochemical identity with crustecdysone. *Tetrahedron Lett.*, **24**: 1113–1114.

Jensen, E. V., and H. I. Jacobson, 1962. Basic guides to the mechanism of estrogen action. *Recent Progr. Hormone Res.*, **18**: 387–414.

Karlson, P., and C. Bode, 1969. Die Inaktivierung des Ecdysons bei der Schmeissfliege *Calliphora erythrocephala* Meigen. *J. Insect Physiol.*, **15**: 111–118.

Karlson, P., C. E. Sekeris and R. Maurer, 1964. Verteilung von Tritium-markiertem Ecdyson in Larven von *Calliphora erythrocephala*. *Hoppe-Seylers Z. Physiol. Chem.*, **336**: 100–106.

King, D. S., and J. B. Siddall, 1969. Conversion of α-ecdysone to β-ecdysone by crustaceans and insects. *Nature*, **221**: 955–956.

Levenbook, L., 1958. Intracellular water of larval tissues of the southern armyworm as determined by the use of C^{14}-carboxy-inulin. *J. Cell. Comp. Physiol.*, **52**: 329–339.

Moriyama, H., D. S. King, K. Nakanishi, T. Okauchi, J. B. Siddall, and W. Hafferl, 1970. On the origin and metabolic fate of α-ecdysone in insects. *Gen. Comp. Endocrinol.*, in press.

Nakanishi, K., M. Koreeda, M. L. Chang and H. Y. Hsu, 1968. Insect hormones. V. The structures of ponasterones B and C. *Tetrahedron Lett.*, **24**: 1105–1110.

Ohtaki, T., and C. M. Williams, 1970. Inactivation of α-ecdysone and cyasterone by larvae of the fleshfly, *Sarcophaga peregrina*, and pupae of the silkworm, *Samia cynthia*. *Biol. Bull.*, **138**: in press.

Ohtaki, T., R. D. Milkman and C. M. Williams, 1968. Dynamics of ecdysone secretion and action in the fleshfly *Sarcophaga peregrina*. *Biol. Bull.*, **135**: 322–334.

Takemoto, T., Y. Hikina, H. Hikino, W. Ogawa and N. Nishimoto, 1968. Structure of rubrosterone, a novel C_{19} metabolite of insect-moulting substances from *Achranthes rubrofusca*. *Tetrahedron Lett.*, **24**: 3053–3056.

Thompson, M. J., J. N. Kaplanis, W. E. Robbins and R. T. Yamamoto, 1967. 20,26-dihydroxyecdysone, a new steroid with moulting hormone activity from the tobacco hornworm, *Manduca sexta* (Johannson). *Chemical Communications*, **1967**: 650–653.

Weevers, R. DeG., 1966. A lepidopteran saline: effects of inorganic cation concentrations on sensory, reflex and motor responses in a herbivorous insect. *J. Expl. Biol.*, **44**: 163–175.

Williams, C. M., 1970. Hormonal interactions between plants and insects. *In:* E. Sondheimer and J. Simeone, Eds., *Chemical Ecology*. Academic Press, New York.

Wyatt, G. R., 1968. Biochemistry of insect metamorphosis. *In:* W. Etkin and L. Gilbert, Eds., *Metamorphosis: A Problem in Developmental Biology*. Appleton-Century-Crofts, New York.

Reference: *Biol. Bull.*, 138: 129–137. (April, 1970)

THE EFFECT OF ACTH ON THE SLOUGHING FREQUENCY OF NORMAL AND THYROIDECTOMIZED *ANOLIS CAROLINENSIS* (IGUANIDAE, LACERTILIA)

K. W. CHIU[1] AND W. G. LYNN

Department of Biology, The Catholic University of America, Washington, D. C. 20017

Periodic shedding of the surface layer of the skin is a characteristic feature of squamate reptiles that has long been known to be controlled or regulated by hormone action (see reviews: Lynn, 1970; Maderson, Chiu and Phillips, 1970). Since the early work of Drzewicki (1926) and Eggert (1933, 1936) the thyroid has been regarded as the most important endocrine gland involved in this phenomenon. These authors found that in the lizard, *Lacerta,* thyroidectomy results in a cessation of molting and thyroidectomized animals with thyroid tissue implanted in the back muscles are able to carry out at least one or two successful molts. Later studies have confirmed the conclusion that the process of ecdysis in lizards is influenced by the thyroid hormone although there are some species in which thyroidectomy causes only a lengthening of the period between molts rather than a complete inhibition of sloughing (*Hemidactylus*, Noble and Bradley, 1933). For snakes the evidence indicates that the thyroid is also important in the control of the shedding process but acts quite differently; thyroidectomy results in an increased frequency of molting and thyroid administration inhibits it (Schaefer, 1933; Halberkann 1953, 1954; Chiu and Lynn, 1970).

However, it is clear that other hormones than those of the thyroid gland may also be involved in skin shedding in reptiles. It has been shown that administration of adrenocorticotrophic hormone (ACTH) inhibits sloughing in the snake, *Natrix* (Halberkann, 1954; Goslar, 1958) and this hormone has proved similarly effective in hypophysectomized lizards, *Gekko* (Chiu and Phillips, unpublished results). Prolactin administration on the other hand, is reported to increase the sloughing rate in *Anolis* (Maderson and Licht, 1967).

The present study is an examination of the effects of ACTH on the sloughing rate in *Anolis* and on the histological changes in the skin during the sloughing process.

MATERIALS AND METHODS

A number of shipments of adult *Anolis carolinensis* of both sexes were obtained from May to October, 1968 from a supply company in Louisiana. The specimens were kept in groups of 5 to 10 animals in aquaria measuring 16 × $10\frac{1}{2}$ × $8\frac{1}{2}$ inches at 32 ± 1° C with 7 hours of lighting daily. They were given larvae and adults of

---

[1] Present address: Department of Biology, Chung Chi College, The Chinese University, Hong Kong.

Tenebrio and the animals were individually observed during each feeding period to make sure that all took food. Water was available at all times. Before any animal was used in the experiment, it was allowed to go through at least two consecutive sloughs so that the length of the pre-experimental sloughing cycle could be ascertained.

The effect of ACTH on shedding was investigated in the following groups: (a) normal, intact, untreated lizards (5 animals); (b) thyroidectomized lizards (9); (c) sham thyroidectomized lizards (5); (d) intact animals receiving intraperitoneal (i.p.) injections of 0.4 I.U. ACTH (Nutritional Biochemical Corp.) in 0.05 ml of a dilute gelatin solution on alternate days (9); (e) intact animals receiving alternate day i.p. injections of 0.05 ml of the gelatin solution used as vehicle in group d (5); (f) thyroidectomized lizards receiving i.p. injections of 0.4 I.U. ACTH in gelatin solution on alternate days (6) and (g) thyroidectomized animals receiving alternate day i.p. injections of 0.05 ml of the gelatin solution used as vehicle for group f (3).

Thyroidectomy was performed under anesthesia with sodium barbital (Nembutal, Abbott) at about 2 mg per 100 g body weight. A transverse incision was made at each side of the ventral surface of the neck anterior to the pectoral girdle, and the two lobes of the thyroid gland were removed. Sham-thyroidectomy was performed in a similar way except that the operation was carried only to point of exposing the gland. The incisions usually healed within a few days.

Thyroidectomy or sham-thyroidectomy and/or administration of ACTH was always performed on the day the animal sloughed. The experiment was terminated at the end of the second slough after treatment in those animals which subsequently sloughed or at the end of at least 60 days in those which did not.

Changes in the epidermis during the sloughing cycle were followed by taking biopsy samples of digits from all animals at roughly weekly intervals. These were fixed in Bouin's fluid, and processed for histological examination using the method described by Maderson and Licht (1967) and Lilywhite and Maderson (1968).

A further set of experiments was performed to assess the effect of ACTH on thyroid activity in the intact animals. A new batch of animals, obtained in February 1969, was kept under identical conditions as the previous lot, and was divided into three groups. (1) normal, intact, untreated controls (5 animals); (2) intact animals receiving i.p. injections of 0.4 I.U. of ACTH in 0.05 ml of gelatin solution daily (5); (3) intact animals receiving i.p. injections of 0.05 ml of the gelatin vehicle daily. The status of the thyroid activity at the end of 7 days was checked by administration of 5 μC of carrier-free I^{131} and measuring thyroidal uptake of the isotope during the subsequent 7 days using equipment and methods described elsewhere (Lynn, McCormick and Gregorek, 1965). At the end of the counting period, the animals were sacrificed and the thyroid glands were processed for histological examination.

RESULTS

General observations

The mortality rate of the lizards in the laboratory was high during the pre-experimental sloughing cycle. It was noted that many animals gradually refused mealworms and partially digested mealworms were excreted. It is probable that keeping *Anolis* at a constant environmental temperature of 32 ± 1° C and/or feeding this lizard with a monotonous diet of mealworms probably has a detrimental effect on the animal. After treatment was started, some experimental animals died within a period of less than 60 days (26 to 56) during which period no sloughs occurred and these, of course, are not represented in the sloughing records presented in Tables I and II. They came from the following groups; sham thyroidectomized of group c (1 animal); thyroidectomized of group b (6); thyroidectomized receiving ACTH of group f (2); intact animals injected with ACTH of group d (4). With the exception of thyroidectomized animals receiving ACTH (group f) and two thyroidectomized, vehicle controls (group g), all remaining animals sloughed at least once during the experiment. In normal, intact, untreated controls (group a), sham-thyroidectomized (group c) and intact, vehicle controls (group e) the process of ecdysis was always complete and required only a few hours. In thyroidectomized lizards and in intact ACTH-treated animals (groups b and d) sloughing was seldom complete. It was usually first indicated by small pieces of shed skin on the digits and the outer epidermal layers of the digit were often shed while the rest of the skin surface showed no sign of shedding at all. The time from this first indication of shedding in the digits to complete removal over the whole body surface might then require 8 to 10 days or might be indefinitely prolonged so that the animals never did completely slough. In these latter animals, no subsequent sloughs were recorded. On the backs of these animals, and of the two thyroidectomized animals receiving vehicle injections, small areas of skin surface were shed. These shed pieces were the result of piling up of the alpha keratin layer, and their occurrence cannot be taken as sloughing records (see below).

TABLE I

The length of the sloughing cycle in Anolis *under various treatments*
Mean ± S. E.

Treatment group	No. of animals	Length of sloughing cycle in days		
		Pre experimental	Post treatment	
			1st	2nd
(a) Intact control	5	18.8 ± 0.6	20.6 ± 2.0 (5)	19.4 ± 2.2 (5)
(b) Thyroidectomized	9	22.3 ± 0.9	51.0 ± 12.1 (4)	48.5 ± 7.5 (2)
(c) Sham – thyroidectomized	5	20.8 ± 0.9	23.0 ± 1.2 (4)	22.0 ± 3.5 (3)
(d) Intact + ACTH	9	21.3 ± 2.5	35.3 ± 4.1 (6)*	34.6 ± 3.0 (5)**
(e) Intact + vehicle	5	20.5 ± 2.3	23.5 ± 1.0 (4)	24.8 ± 0.6 (4)
(f) Thyroidectomized + ACTH	6	32.0 ± 5.7	51.0 (1)	26.0 (1)
(g) Thyroidectomized + vehicle	3	41.3 ± 9.8	35.0 (1)	46.0 (1)

Figures in parentheses refer to number of recorded sloughs after treatment.
* Group d vs. group e, $P < 0.05$.
** Group d vs. group e, $p < 0.02$.

Effect on the length of the sloughing cycle (Table I).

Examination of the figures for the pre-experimental cycles given in Table I shows that the cycles for the first five groups (a through e) agree closely while those for the last two groups (f and g) are longer and more variable. This is probably a seasonal difference since experiments a to e were begun in May and June while experiments f and g started in October. However, it will be seen that there is no statistically significant difference in the length of the pre-experimental cycle immediately prior to treatment between various experimental groups (b, d, f) and their controls (c, e, g). Nor is there any significant difference between the lengths of these cycles and those of the experimental cycles in normal, intact, untreated animals (group a), sham-thyroidectomized (group c) and the intact control animals (group e). This indicates that neither sham-thyroidectomy nor vehicle injection have any effect on the length of the sloughing cycle.

A statistically significant increase in the length of the sloughing cycle of about 50% in the ACTH-treated intact animals (group d) was found when compared with the vehicle controls (group e), the normal, untreated controls (group a) and the pre-experimental cycles. A greater increase of the cycle length (about 100%)

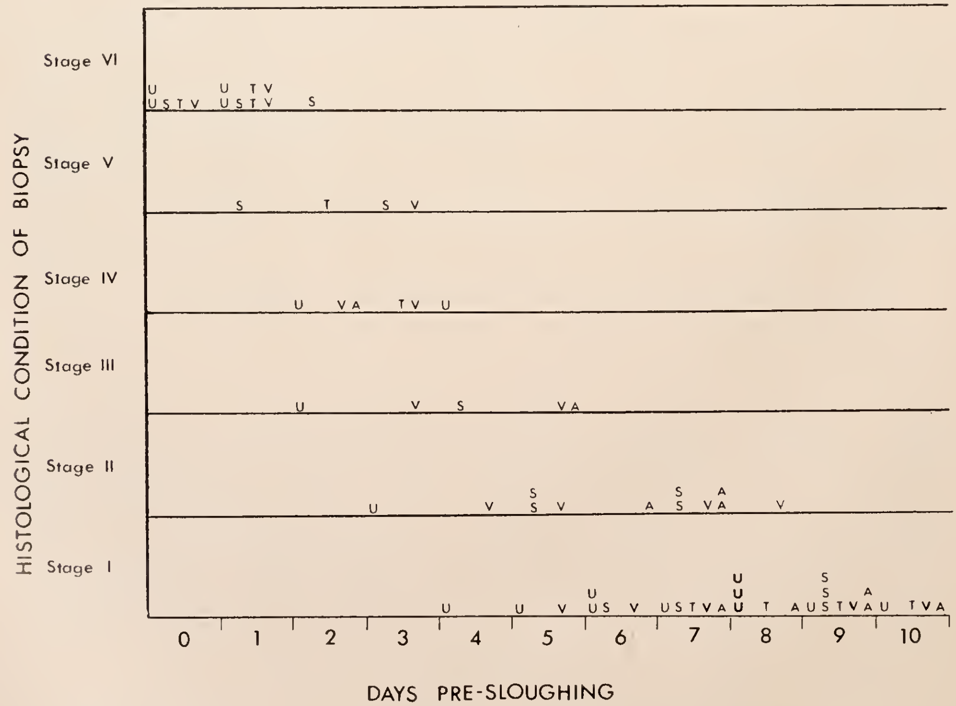


FIGURE 1. Scatter diagram showing the rates of differentiation of the inner epidermal generation during the renewal phase in various groups of *Anolis carolinensis*. U untreated control; S sham-thyroidectomized; T thyroidectomized; V intact plus vehicle; A intact plus ACTH.

was found in four thyroidectomized animals which sloughed (group b) and one thyroidectomized animal receiving vehicle administration (group g) as compared with the sham-operated controls (group c). No slough was noted in ACTH-treated thyroidectomized animals (group f) except in one animal which, on autopsy, was found to have some ectopic thyroid follicles in the mid-ventral line of the neck.

Effect on the epidermis

Histological changes in the epidermis in control animals are similar to those described previously (Maderson and Licht, 1967; Lilywhite and Maderson, 1968) and can be divided into a resting phase and a renewal phase. The lengths of these two phases are comparable among the control groups except in the untreated controls (group a) which appear to have a shorter renewal phase (Fig. 1). In ACTH-treated intact animals (group d) and thyroidectomized animals with or without administration of vehicle (groups g and b) which sloughed, the resting phase is lengthened, and it is indefinitely prolonged in those that failed to slough. Biopsy digit samples taken from those few animals which sloughed indicate that there is no difference in the rate of differentiation of the epidermis in the digit, during the renewal phase as compared with that of the control animals. In both cases the renewal phase occupies about one week. However, since shedding over the general body surface of the ACTH-treated or thyroidectomized animals does not occur synchronously with that on the digits the *total* period of time occupied by the renewal phase in the sloughing cycle is longer as compared with control animals.

TABLE II

The percentage uptake of I^{131} by the thyroid gland in Anolis

Mean ± S. E.

Treatment group	% uptake of I^{131}						
	1st	2nd	3rd	4th	5th	6th	7th day
(1) Intact control	20.6 ±5.4	30.2 ±6.5	32.8 ±6.6	33.6 ±6.5	31.9 ±6.5	30.4 ±7.3	31.0 ±6.1
(2) Intact +ACTH	16.4 ±3.6	24.0 ±5.1	27.3 ±5.6	28.7 ±5.5	29.8 ±5.6	29.6 ±6.1	28.3 +5.6
(3) Intact +vehicle	17.8 ±6.2	28.5 ±10.4	28.5 ±8.7	28.4 ±8.3	27.2 ±8.6	26.2 ±8.4	25.4 ±7.5

In animals that failed to shed, the resting phase is the only phase seen in all biopsies. Samples taken during the first two weeks after ACTH treatment show a typical "resting phase" of the epidermis (Maderson, 1966), in which there is a thin alpha keratin layer lying on top of the stratum germinativum with no cell layers between. Subsequent biopsies, except those taken from thyroidectomized

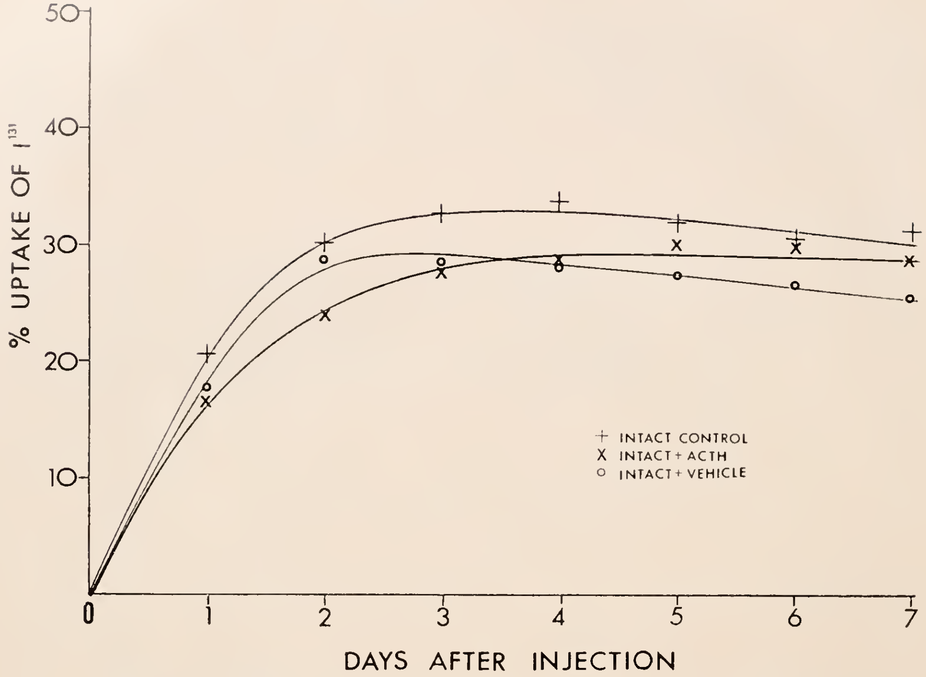


FIGURE 2. The uptake of I^{131} by the thyroid gland after administration of 5 μC of carrier-free I^{131}.

animals injected with ACTH, show a varying number of cell layers above the stratum germinativum and an increase in the thickness of the alpha keratin layer. The stratum germinativum also changes from cuboidal to columnar. In thyroidectomized animals receiving ACTH, no sign of proliferative activity of the germinative layer or increase of the alpha keratin layer can be found.

Effect of ACTH on thyroid activity (Table II and Fig. 2)

There is no significant difference between the percentage uptake of I^{131} in the three groups of intact animals with or without ACTH vehicle (groups 1, 2, and 3). Moreover there is no difference in the histological appearance of the thyroid gland in these animals. This finding suggests that ACTH probably has no influence on thyroid activity in the lizard.

DISCUSSION

It is well known that the thyroid hormones play an important part in the control of ecdysis in lizards. Removal of the thyroid results in complete inhibition of sloughing in the lacertid lizard *Lacerta* (Sembrat and Drzewicki, 1936; Eggert, 1936). However in gekkonids (*Hemidactylus,* Noble and Bradley, 1933; *Gekko,* Chiu *et al.* 1967) thyroidectomy causes only a decrease in sloughing frequency. In

Anolis, an iguanid, earlier reports indicate that thyroid removal or administration of thyroid inhibiting drugs results only in lengthening the sloughing cycle rather than preventing it (Ratzersdorfer *et al.*, 1949; Adams and Craig, 1951). In the present study it was found that half of the thyroidectomized animals showed a decrease in sloughing frequency while the other half failed to shed at all during the period of observation. It seems probable that these diverse results may indicate that, while thyroid function may be the primary controlling agent in skin shedding in lizards, it is not the only factor involved. Several recent studies indicate the significance of other hormonal factors. For example, the hypophysis has been shown to have a role in the sloughing process in lizards and it has been suggested that hypophyseal hormones may have a direct effect on the epidermis (Maderson and Licht, 1967; Chiu and Phillips, unpublished results). Gonadotropins do not influence sloughing in *Anolis* but prolactin causes an increase in sloughing rate (Maderson and Licht, 1967). ACTH has been found to inhibit skin shedding in hypophysectomized *Gekko* (Chiu and Phillips, unpublished results) and the present study shows that administration of ACTH lengthens the sloughing cycle in both normal and thyroidectomized *Anolis.* Furthermore, that this inhibitory action of ACTH is a direct one rather than one intermediated by the thyroid gland is shown by the second series of experiments which indicate that this hormone has no effect upon the thyroidal uptake of radioiodine or upon thyroid histology. It is also unlikely that the effect of ACTH upon ecdysis is mediated through the adrenal gland for it has been demonstrated that administration of adrenocorticoids has no effect on sloughing in squamate reptiles (Halberkann, 1954; Chiu and Phillips, unpublished results).

The effects of various hormones on epidermal changes during the sloughing cycle have been investigated in a number of lizards (Eggert, 1936; Sembrat and Drzewicki, 1936; Adams and Craig, 1951; Chiu *et al.*, 1967; Maderson and Licht, 1967; Chiu and Phillips, unpublished results). While no abnormality in epidermal histology has been noted in *Gekko* under various endocrine manipulations (Chiu *et al.*, 1967; Chiu and Phillips, unpublished results), or in *Anolis* under prolactin administration (Maderson and Licht, 1967), an increase in the thickness of the keratin layer after thyroidectomy (*i.e.*, hyperkeratosis) has been reported in *Lacerta* (Eggert, 1936; Sembrat and Drzewicki, 1936) and in *Anolis* (Adams and Craig, 1951). In the present study such an increase in thickness of the alpha keratin layer was noted about 40 days after sloughing in thyroidectomized animals that have greatly protracted cycles and in those that do not slough at all. This finding confirms Adams and Craig's (1951) study on *Anolis.* However we have also found this phenomenon in ACTH-treated intact animals. We would suggest hyperkeratosis of the alpha keratin layer is probably a general feature in any protracted cycle in *Anolis.* It has been seen that proliferation of the stratum germinativum continues in thyroidectomized animals and also in non-thyroidectomized animals treated with ACTH. A delay in the appearance of the renewal phase after thyroidectomy and subsequent keratinization of the newly formed cells would result in an increase in thickness of the alpha keratin layer. It is understandable that no increase of keratin layers is observed in thyroidectomized animals receiving ACTH since no proliferative activity of the germinative layer of the epidermis is evident in these animals. We may conclude that thyroidectomy delays differentiation of the epidermal cell layers, and that ACTH has an inhibitory effect on cellular proliferation in the epidermis.

We are grateful to Pauline Chiu for her technical assistance.

SUMMARY

The effect of thyroidectomy and administration of ACTH (0.4 I.U. on alternate days) on sloughing performance in normal and thyroidectomized *Anolis* has been investigated. Either thyroidectomy or injection of ACTH decreases the frequency of sloughing, but does not inhibit it. ACTH administration and thyroidectomy together prevent sloughing. Histological study of the skin shows that the resting phase of the sloughing cycle is lengthened in thyroidectomized animals and in intact animals receiving ACTH injections, and is indefinitely prolonged in thyroidectomized animals injected with ACTH. Hyperkeratosis (increase in the thickness of the keratin layer) is observed in all animals with a protracted cycle, except in thyroidectomized animals with ACTH administration. Since there is no difference in the I^{131} uptake or in the histological appearance of the thyroid gland following ACTH treatment, it is suggested that ACTH influences the sloughing cycle independently of the thyroid gland probably through a direct inhibitory effect on cellular proliferation.

LITERATURE CITED

ADAMS, A. E., AND M. CRAIG, 1951. The effects of antithyroid compounds on the adult lizard, *Anolis carolinensis*. *J. Exp. Zool.*, **117**: 287–315.

CHIU, K. W., AND W. G. LYNN, 1970. The role of the thyroid in skin-shedding in the shovel-nosed snake, *Chionactis occipitalis*. *Gen. Comp. Endocrinol.*, in press.

CHIU, K. W., J. G. PHILLIPS AND P. F. A. MADERSON, 1967. The role of the thyroid in the control of the sloughing cycle in the Tokay (*Gekko gecko*, Lacertilia). *J. Endocrinol.*, **39**: 463–472.

DRZEWICKI, S., 1926. Influence de l'extirpation de la glande thyroide sur la mue du lézard (*Lacerta agilis* L.). *C. R. Séanc. Soc. Biol.* **95**: 893–895.

EGGERT, B., 1933. Ueber die histologischem und physiologischen Beziehungen zwischen Schilddrüse und Häutung bei den einheimischen Eideschen. *Zool. Anz.*, **105**: 1–9.

EGGERT, B., 1936. Zur Morphologie und Physiologie der Eideschsenschilddrüse. 3. Über die nach entfernung der Schilddrüse auftretenden allgemeinen Ausfallserscheinungen und über die Bedeutung der Schilddrüse für die Häutung und für die Kaltestarre. *Z. Wiss. Zool.*, **148**: 221–260.

GOSLAR, H. G., 1958. Die Reptilienhaut als endokrines Testobjekt. *Endokrinologie*, **36**: 279–286.

HALBERKANN, J., 1953. Untersuchungen zur Beeinflussung des Häutungszyklus der Ringelnatter durch Thyroxin. *Arch. Dermatol. Syphilol.* **197**: 37–41.

HALBERKANN, J., 1954. Zur hormonalen Beeinflussung des Häutungszyklus der Ringelnatter. *Z. Naturforsch.*, **96**: 77–80.

LILYWHITE, H. G., AND P. F. A. MADERSON, 1968. Histological changes in the epidermis of the subdigital lamellae of *Anolis carolinensis* during the shedding cycle. *J. Morphol.*, **125**: 379–402.

LYNN, W. G., 1970. The thyroid. *In:* A. D'A Bellairs, C. Gans and E. Williams, eds., *The Biology of the Reptilia*. Academic Press, New York, in press.

LYNN, W. G., J. J. MCCORMICK AND J. C. GREGOREK, 1965. Environmental Temperature and thyroid function in the lizard, *Anolis carolinensis*. *Gen. Comp. Endocrinol.*, **5**: 587–595.

Maderson, P. F. A., 1966. Histological changes in the epidermis of the Tokay (*Gekko gecko*) during the sloughing cycle. *J. Morphol.,* **119**: 39–50.

Maderson, P. F. A., K. W. Chiu and J. G. Phillips, 1970. Endocrine-epidermal relationships in squamate reptiles. *Mem. Soc. Endocrinol.,* in press.

Maderson, P. F. A., and P. Licht, 1967. Epidermal morphology and sloughing frequency in normal and prolactin treated *Anolis carolinensis* (Iguanidae, Lacertilia). *J. Morphol.,* **123**: 157–171.

Noble, G. K., and H. T. Bradley, 1933. The relation of the thyroid and the hypophysis to the molting process in the lizard, *Hemidactylus brookii. Biol. Bull.,* **64**: 289–298.

Ratzersdorfer, C., A. S. Gordon and H. A. Charipper, 1949. The effects of thiourea on the thyroid gland and molting behavior of the lizard, *Anolis carolinensis. J. Exp. Zool.,* **112**: 13–27.

Schaefer, W. H., 1933. Hypophysectomy and thyroidectomy of snakes. *Proc. Soc. Exp. Biol. Med.,* **30**: 1363–1365.

Sembrat, K., and S. Drzewicki, 1936. The influence of the selachian thyroid upon the molting process of the lizards, with some remarks on the skin, the eyes, and the ultimobranchial body of the thyroidectomized lizards. *Zool. Pol.* **1**: 119–169.

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

Reference: *Biol. Bull.*, 138: 138–156. (April, 1970)

POPULATION GENETICS OF MARINE SPECIES OF THE PHYLUM ECTOPROCTA

JAMES L. GOOCH AND THOMAS J. M. SCHOPF

Department of Biology, Juniata College, Huntingdon, Pennsylvania 16652 and Department of the Geophysical Sciences, University of Chicago, Chicago, Illinois 60637

The literature of marine invertebrate zoology contains relatively few references to genetic studies. Buzzati-Traverso, in a summary of accomplishments and a prospective future of marine biology, declared that knowledge of the genetics of marine organisms was in its infancy and that an understanding of evolutionary processes in the ocean required much more knowledge in that area (Buzzati-Traverso, 1960). Only a few investigations have detected unit genes in the Mendelian sense in which alleles with contrasting phenotypic expression were shown to segregate in the progeny. A partial list of works of this type includes those on the amphipod *Gammarus chevreuxi* (Sexton and Clark, 1936), the copepod *Tisbe reticulata* (Bocquet, 1951; Battaglia, 1958), the isopod *Jaera marina* (Bocquet, 1953), the polychaete *Pomotoceros triqueter* (Føyn and Gjøen, 1954), and the ascidian *Botryllus schlosseri* (Sabaddin, 1959; Milkman, 1967). Most investigations began by noting the occurrence of color polymorphism in the species concerned and led to the elucidation of a simple genetic controlling mechanism. A few other aspects of marine genetics have received brief attention. The cytogenetics of the gastropod *Thais lapillus* in different environments was studied (Staiger, 1954), and the polygenic basis of sex ratios in the copepod *Tigriopus* was investigated (Ar-Rushdi, 1958). Barigozzi (1960) speculated on the nature of genetic systems in sessile marine organisms. Useful reviews of the state of marine genetics have been published (Buzzatti-Traverso, 1960; Ray, 1960; Battaglia, 1965). The chief conclusion to be gained is that genetic studies on marine animals have been extremely limited in scope and theoretical significance.

Other than chromosome counts of a few species (Makino, 1951), nothing is known of the genetics of the Phylum Ectoprocta. Marine ectoprocts are abundant, sessile benthic animals that feed on phytoplankton by means of a ciliated crown of tentacles, the lophophore. The initial phase is a solitary planktonic larva, which soon settles. A colony is formed by successive asexual budding of new individuals from previously formed individuals. Thus each individual (zooid) of an ectoproct colony is genetically identical.

Several fundamental aspects of the life cycle of species of ectoprocts are poorly known. A point of special confusion has involved the mode of fertilization because the majority of ectoprocts are hermaphroditic. According to Hyman (1959), most specialists believe that hermaphroditic ectoprocts are self-fertilizing. Silén (1966) and Bullivant (1967) concluded from observations of sperm release into sea water that at least some species may practice outbreeding. Resolution of the question of either autogamy or intra-colony selfing *versus* outbreeding, and the understanding of genetic systems in general have been impeded by two practical difficulties. (1) Morphological variants of potential genetic origin have not been recognized in any ectoproct species; and (2) ectoprocts have not been successfully crossed in the laboratory. One or both of these difficulties apply equally well to many marine animals.

For the last decade, and especially since 1966 when the papers of Hubby and Lewontin, and Lewontin and Hubby appeared, electrophoretic and histochemical-staining techniques have been utilized for the detection of genetic variants at single loci. Crude protein extracts are prepared from individual organisms and spotted onto starch or polyacrylamide gels. Polypeptides coded by each allele of a locus migrate a characteristic distance in an electric field applied across the gel. The mobility of the polypeptide depends on its electrical charge, size and conformation. Mutations that alter these properties of a polypeptide will alter its mobility; therefore the number and kinds of alleles at a locus can often be determined by the electrophoretic pattern displayed by the corresponding polypeptides after fixation and staining by the proper histochemical means.

A considerable body of literature on electrophoretic variants of enzymes now exists and much of it concerns genetic variation (reviewed by Shaw, 1965). The use of electrophoresis in population genetics has been strikingly exploited in studies of *Drosophila* (for example, Hubby and Lewontin, 1966; Lewontin and Hubby, 1966; Johnson, Kanapi, Richardson, Wheeler and Stone, 1966; Yarbrough and Kojima, 1967; Kojima and Yarbrough, 1967; Stone, Wheeler, Johnson and Kojima, 1968; O'Brien and MacIntyre, 1969). A beginning has been made in the application of these techniques to other organisms, most notably by Manwell in taxonomic problems of marine invertebrates (Manwell, 1966; Manwell and Baker, 1963, 1968; Manwell, Baker, Ashton and Corner, 1967). Much of the protein variation in his research was ascribed to genetic causes, but gene loci were not usually identified.

We used electrophoretic techniques to identify gene loci in local populations of several species of marine ectoprocta. Results for the two species best known are reported here. Where our collections were sufficient, gene and genotype frequencies were determined for individual loci, and the amount of genetic variation was calculated.

Bugula stolonifera is erect and foliaceous in growth form, and is lightly calcified, whereas *Schizoporella unicornis* grows as an encrusting, highly calcified sheet (Schopf and Manheim, 1967, 1968). Each of these species is widely reported from the Atlantic Coast of North America, England, and Western and Southern Europe (Ryland, 1960, 1965; Maturo, 1966).

MATERIALS AND METHODS

Ectoproct colonies were collected in the vicinity of Woods Hole, Massachusetts. *Schizoporella unicornis* was obtained from pilings of the Marine Biological Laboratory (MBL) dock facing into Vineyard Sound, from a floating dock in Green Pond, Massachusetts, 10 km east of Woods Hole, and from a dredge haul from Vineyard Sound about 35 km southwest of Woods Hole (see Fig. 6). Most of the *Bugula stolonifera* came from metal drums used as floats for the MBL supply department area in Eel Pond, Woods Hole. Supplementary collections were taken from Green Pond.

Colonies were kept alive in running seawater tanks. They were prepared for electrophoresis by first removing any intimately associated fauna, such as amphipods and nematodes from *Bugula,* and overgrown barnacles, tunicates and bivales from *Schizoporella.* Portions of *Bugula* colonies containing 1000–1500 living zooids were triturated in a tissue grinder in cold $12\frac{1}{2}\%$ sucrose buffer. About 500 zooids per colony of the much more heavily calcified *Schizoporella* were ground in sucrose-buffer in an Albers micromortar (Thomas Co.). Ground samples were centrifuged at room temperature for 15 minutes at 3500 rpm in

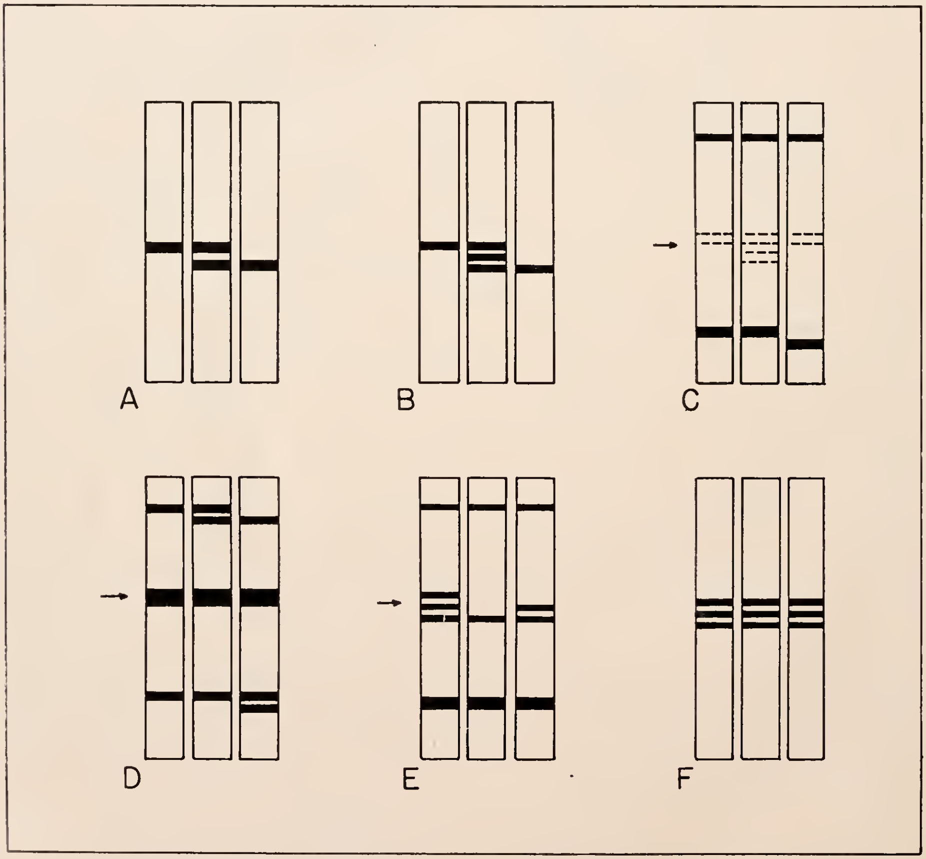


FIGURE 1. Diagrammatic sketch showing patterns of bands illustrating genetic loci: A. Simple diallelic system with an upper band homozygote, a heterozygote, and a lower band homozygote; B. Diallelic system with an upper band homozygote, a heterozygote with a hybrid band, and a lower band homozygote; C. Inhibition of middle zone without affecting upper or lower zones; D. Monomorphic middle zone which does not vary in position relative to zones of defined loci above and below it; E. Polymorphic middle zone which varies in position relative to zones of defined loci above and below it; F. Zone of several bands of possibly more than one locus but which cannot be further resolved by techniques at hand. Arrows denote the zone under consideration.

1 ml polyethylene centrifuge tubes and 10 μl of supernatant were pipetted onto the gels.

Electrophoresis was performed with a vertical electrophoresis cell (E-C Corporation, Philadelphia, Pennsylvania) similar to the method of Hubby (1963) and Hubby and Lewontin (1966). However, a 6½% polyacrylamide gel was used throughout because of its superior resolution of bands of malate dehydrogenase. Electrophoresis was conducted for 3 hours at 350–400 V, and 50–100 mA for most analyses. Recirculating water from an ice bath kept the buffer temperature near 15° C. Usually 23 colonies were electrophoresed simultaneously. The 24th slot on the gel was reserved for Bovine serum albumin (Nutritional Biochemicals Corp.). The major, fast moving band of Bovine albumin served as a reference standard for distance in all analyses.

Electrophoresis of nonspecific esterases was conducted using 0.2 M Tris-glycine buffer, pH 8.3. Gels were preincubated in 150 ml of 0.5 M boric acid at 5° C for one hour and then transferred to 150 ml of 0.1 M phosphate buffer solution, pH 6.5 at room temperature, containing 40 mg of α-naphthyl acetate as substrate and 75 mg of Fast Red TR Salt as dye coupler. Staining was complete in two hours, after which gels were photographed and described, and then preserved in 4% acetic acid.

Esterase inhibition studies were conducted on 3–5 colonies with eserine sulfate, EDTA, and phenylmethylsulfonylfluoride (PMSF). In alternate substrate studies, 40 mg β-naphthyl acetate and 0.3 ml α-naphthyl butyrate were substituted for the usual substrate.

Determinations of malate dehydrogenase were made using 0.2 M Tris-glycine buffer, pH 8.3. Tris-borate buffer, pH 8.9, and Tris-EDTA-borate buffer, pH 8.9, were also tried but did not yield good resolution. After electrophoresis, gels were incubated in 150 ml of 0.005 M Tris-HCl-buffer, pH 8.5 at room temperature, containing 20 mg of NAD, 60 mg of Nitro BT chloride and 3 mg of phenazine methosulfate. Bands were developed after 1½–2½ hours of incubation in the dark.

Electrophoretic determinations of "leucine" aminopeptidase were made with 0.1 M Tris-borate buffer, pH 8.9, containing 1mM $MgCl_2$. Following a preincubation period of 30 minutes in 125 ml 0.1 M Tris-maleate buffer, pH 5.3, and 40 mg of Leucyl-β-naphthylamide at room temperature, 75 mg of Fast Black K salt (Pfaltz and Bauer, Co., New York) were added. Bands developed during the following 1–2 hours. Occasionally it was necessary to remove the old staining solution and add fresh substrate and dye coupler to permit development of faint bands.

Identification of Genetic Loci

In order to obtain genetic information from electrophoresis gels, a means must be found to estimate the number of gene loci and the number of alleles per locus from the pattern of enzymatically active bands. Much of the difficulty of interpretation is obviated if organisms suspected of carrying different allelic combinations at a locus are crossed under controlled laboratory conditions. The progeny are then scored for patterns and these are compared to expected genotypes. Most discoveries of electrophoretic variants under genetic control have been made in maize, *Drosophila, Mus,* and other organisms that are readily crossed (Shaw, 1965). Many marine invertebrates, and ectoprocts in particular, have never been successfully crossed in the laboratory. Therefore three criteria that do not involve crosses were utilized in this study in estimating the number of genetic loci (Fig. 1).

(1) A codominant diallelic or triallelic locus in which each allele produces a single enzyme band of characteristic mobility is easily recognized (Fig. 1A). Heterozygotes have the bands of both alleles. Each heterozygote band is usually lighter than the corresponding band of the homozygotes since it is produced by a single dose of the gene. This type of band pattern has been noted repeatedly in reference in *Drosophila* and other organisms (see, for example, Wright, 1963; Beckman and Johnson, 1964a; 1964b; Scandalios, 1969). It also occurs in the ectoproct species under study, particularly in esterase gels of *Bugula stolonifera* and LAP gels of both species. We regard such a pattern as a reliable criterion for the existence of a polymorphic genetic locus.

In multimeric enzymes, polypeptide subunits may form one or more intermediate bands approximately evenly spaced between the bands of homozygote mobilities. Commonly a single intermediate band is found in heterozygotes (Fig. 1B) which presents no problem in interpretation. However, the genetic relationships of multimeric enzymes manufactured by several mobility alleles can be complicated (for example, see Burns and Johnson, 1967). Complex loci of this type occur in the species *Bugula turrita* and *B. simplex* (unpublished data) but not in *B. stolonifera* and *S. unicornis*.

(2) The use of specific inhibitors and alternate substrates also permits the recognition of loci. The rationale is that a band or a band complex differentially affected by these agents in comparison to adjacent zones probably includes polypeptides that originate from a single locus (Fig. 1C). This conclusion does not extend to groups of bands with widely different mobilities that happen to respond to the same inhibitors or alternate substrates.

Gels stained for nonspecific esterases with α-naphthyl acetate usually show numerous zones of activity that represent several enzymes. Alternate substrates and various inhibitors with partial specificity have been used to distinguish classes of esterases (Allen, 1961; Ogita and Kasai, 1965; and Manwell *et al.*, 1967). Eserine sulfate inhibits certain cholinesterases, and EDTA inhibits some arylesterases (Augustinsson, 1961). PMSF inhibits acetycholinesterase, trypsin and chymotrypsin (Gold, 1967). The alternate substrates β-naphthyl acetate and α-naphthyl butyrate are preferentially acted upon by certain esterases.

(3) Bands are not randomly scattered over the gels, but are usually localized into discrete zones. A zone is considered representative of a single locus if variation within it appears independent of other zones of the gel (Fig. 1D). Therefore, a zone of activity appearing as a band of uniform mobility is regarded as monomorphic if it is adjacent to zones definitely identified as polymorphic (Hubby and Lewontin, 1966). Similarly, zones containing bands of variable mobility but difficult to interpret genetically are considered polymorphic if they are adjacent to zones identified as either monomorphic or polymorphic by other criteria (Fig. 1E).

We have no criterion for distinguishing loci where series of closely adjacent bands of uniform mobility are present other than the use of inhibitors and alternate substrates as in the case of esterases (Fig. 1F). A series of bands may represent several independent monomorphic loci, a sharing of subunits in a multimeric system or an artifact of conditions of the analysis. In this study, series of adjacent

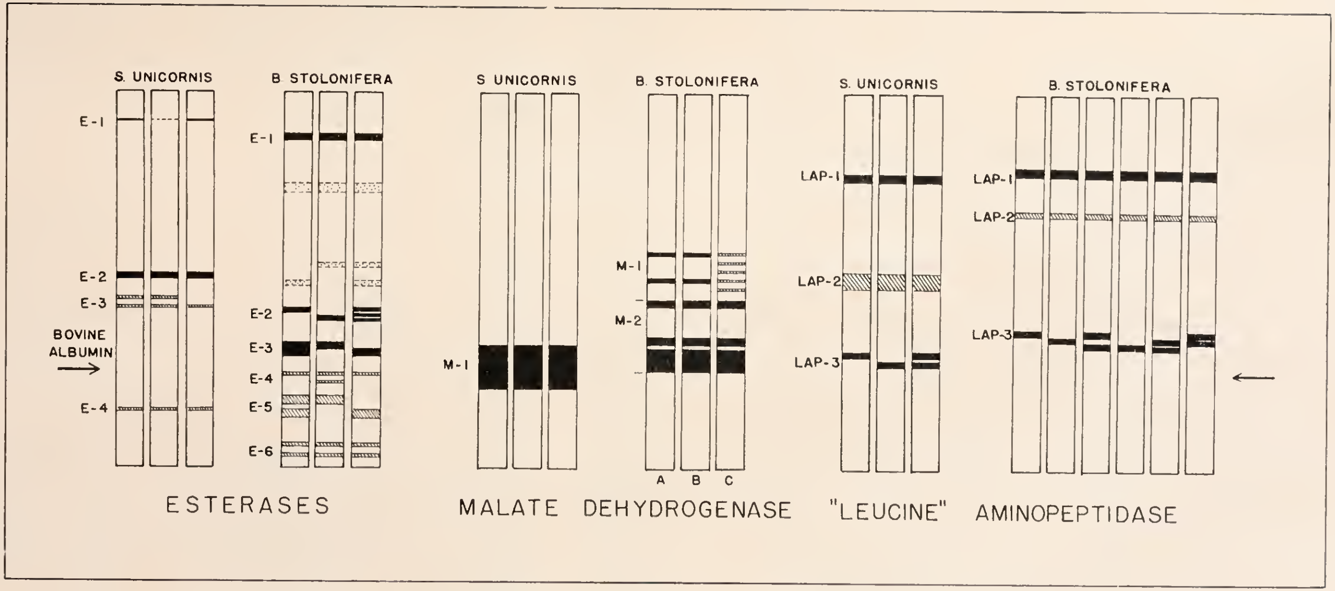


FIGURE 2. Diagram illustrating patterns of banding of *Schizoporella unicornis* and *Bugula stolonifera* in esterases ($n = 48$; $n = 44$, respectively), malate dehydrogenase ($n = 26$; $n = 54$), and "leucine" aminopeptidase ($n = 126$; $n = 43$). Dark bands represent heavily stained zones of enzyme activity, hatched bands stand for more lightly stained zones, and stippled bands faint zones.

bands of uniform mobility have been conservatively regarded as belonging to the same locus.

Based on these determinations we have employed a terminology for enzymes and genetic loci based principally on the system of Hubby and Lewontin (1966). For example, in *B. stolonifera* there are 6 zones of esterase activity under the control of separate genetic loci (Fig. 2). These enzymes are designated esterase-1 through esterase-6 in order of increasing mobility and distance from the origin. The corresponding loci are termed E-1 through E-6. Thus, the fourth zone of esterase activity from the origin is the enzyme esterase-4 and it is controlled by the locus E-4. The esterase-4 enzyme has two mobility variants that migrate distances of 1.01 and 1.04 relative to that of the bovine albumin standard. The mobility variants belong to two alleles of the E-4 locus, and are designated $\text{E-4}^{1.01}$ and $\text{E-4}^{1.04}$ (Table I).

Alleles of some loci produce two or more enzyme bands when homozygous. The mobility of such alleles is designated by a single superscript which is the midpoint between the outermost bands. Thus, the monomorphic locus $\text{M-2}^{.85}$ of *Bugula stolonifera* corresponds to three bands of malate dehydrogenase ranging between 0.75 and 0.95 in mobility.

The number of alleles of the polymorphic loci E-3 in *Schizoporella unicornis* and M-1 in *B. stolonifera* is as yet unknown. Superscripts have not been applied to these loci.

RESULTS

Esterases

Schizoporella unicornis and *Bugula stolonifera* are quite unlike in the number and mobilities of zones of esterase activity (Fig. 2, Table I). The simplest band pattern occurs in *S. unicornis*, in which there are 4 major zones of activity,

TABLE I

Locus and allele nomenclature for esterases and biochemical characterization of loci in Schizoporella unicornis *and* Bugula stolonifera

Species	Locus Monomorphic or Polymorphic	Nomenclature of locus and alleles	Inhibitor			Substrate utilization		
			Eserine	PMSF	EDTA	α-naphthyl acetate	β-naphthyl acetate	α-naphthyl butyrate
Schizoporella unicornis	Monomorphic	$\text{E-1}^{.10}$	++	–	–	+	–	–
	Monomorphic	$\text{E-2}^{.66}$	–	–	–	++	+	++
	Polymorphic	E-3	–	–	–	++	+	+
	Monomorphic	$\text{E-4}^{1.14}$	–	–	–	++	+	+
Bugula stolonifera	Monomorphic	$\text{E-1}^{.16}$	++	–	–	++	+	–
	Polymorphic	$\text{E-2}^{.78}$ $\text{E-2}^{.81}$	–	–	–	+	+	+
	Polymorphic	$\text{E-3}^{.90}$ $\text{E-3}^{.94}$	–	++	–	+	+	+
	Polymorphic	$\text{E-4}^{1.01}$ $\text{E-4}^{1.04}$	+	–	–	+++	++	+
	Polymorphic	$\text{E-5}^{1.11}$ $\text{E-5}^{1.16}$	+	++	–	++	+	+
	Monomorphic	$\text{E-6}^{1.29}$	–	–	–	+	–	–

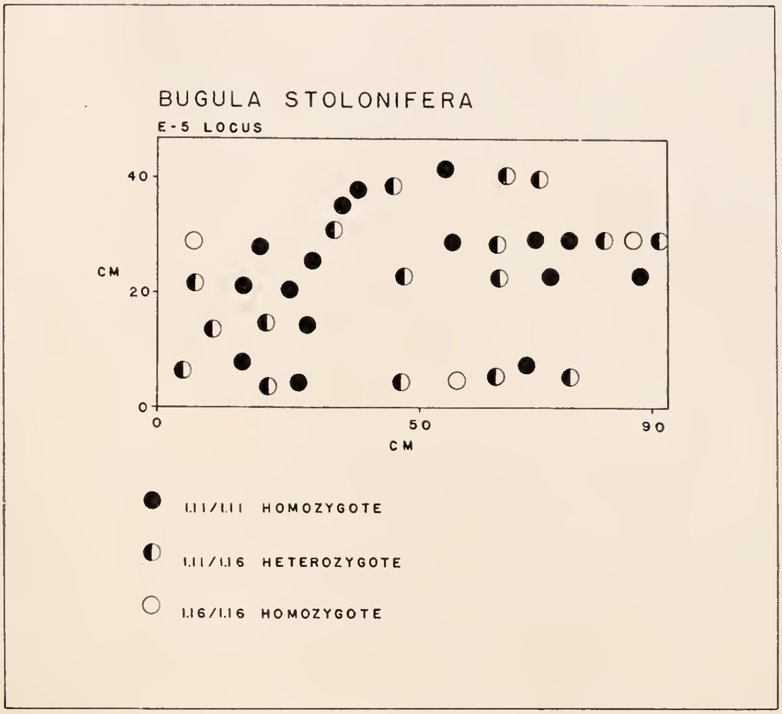


FIGURE 3. Plot of areal distribution of genotypes of *Bugula stolonifera* (locus E-5) on a floating drum at Eel Pond. This pattern of random distribution of a genotype is typical for other loci on this drug and on others.

corresponding to 4 gene loci, 3 monomorphic and 1 polymorphic and diallelic (Table I). Esterase-1, the product of the monomorphic E-1 locus, is inhibited by 1×10^{-4} M eserine sulfate, and does not appear when β-naphthyl acetate and α-naphthyl butyrate are the substrates. Enzymes of the other loci are unaffected by the inhibitors employed and show little differential activity on alternate substrates.

Esterase band patterns are more complex in *B. stolonifera.* The 6 major zones of esterase activity are each controlled by a separate locus, E-1 through E-6. Four of the loci are polymorphic and diallelic (Table I). As in *S. unicornis,* esterase-1 is inhibited by eserine sulfate and does not utilize β-naphthyl acetate and α-naphthyl butyrate. Esterase 3 is inhibited by PMSF and the two bands of esterase-6 are greatly clarified by EDTA.

Malate dehydrogenase

The staining procedure for malate dehydrogenase (MDH) has greater specificity than that for esterases. Nevertheless, several MDH enzymes with different coenzyme and divalent cation requirements are known (Moore and Villee, 1963; Manwell *et al.*, 1967). In our study, attempts to detect NADP- and Mg^{++}-dependent MDH were unsuccessful. NAD-dependent MDH, however, was detected in both species.

In *Schizoporella unicornis*, MDH activity appears in a single band of uniform mobility (Fig. 2). It is the product of the monomorphic locus M-1.

The fastest migrating three bands of *Bugula stolonifera* have constant mobilities in all colonies examined. We conservatively regard them as controlled by the same monomorphic locus, M-2. In contrast, the bands of lesser mobility occur as two variants. In variant *A* (Fig. 2, A, B), two sharp bands with mobilities of 0.58 and 0.68 are present. In variant *B* (Fig. 2, C), the two bands of variant *A* are fainter and are joined by three additional, evenly spaced bands. This 5-banded pattern of variant *B* resembles the band pattern of the heterozygote of a tetramer. It is possible, however, that the variation is non-genetic, as has been noted for various dehydrogenases (see Jacobson, 1968; Thornber, Oliver and Scutt, 1968; and O'Brien and MacIntyre, 1969). On the other hand, the two variants remain clearly recognizable in spite of widely different amounts of crude protein introduced into the gel slots, different parts of the colony electrophoresed (growing apical zooids, reproducing zooids, and degenerating zooids (brown body regions)), and differences in pH and ionic strength of the buffer. This is good evidence that they have a genetic basis. If the variants are genetic, probable differences in gene frequency exist between Eel Pond and Green Pond. Only 4 of 28 colonies (or 14%) from Eel Pond are variant *B* whereas 11 of 26 (42%) from Green Pond are variant *B*. Variants *A* and *B* of *B. stolonifera* are treated as representative of one polymorphic locus, M-1.

TABLE II

Locus and allele nomenclature for malate dehydrogenase and "leucine" aminopeptides in Schizoporella unicornis *and* Bugula stolonifera

Type of protein	Species	Locus Monomorphic or Polymorphic	Nomenclature of locus and alleles
Malate dehydrogenase	*Schizoporella unicornis*	Monomorphic	M-1$^{.99}$
	Bugula stolonifera	Polymorphic	M-1
		Monomorphic	M-2$^{.85}$
"Leucine" aminopeptidase	*Schizoporella unicornis*	Monomorphic	Lap-1$^{.31}$
		Monomorphic	Lap-2$^{.68}$
		Polymorphic	Lap-3$^{.94}$
			Lap-3$^{.98}$
	Bugula stolonifera	Monomorphic	Lap-1$^{.28}$
		Monomorphic	Lap-2$^{.43}$
		Polymorphic	Lap-3$^{.86}$
			Lap-3$^{.88}$
			Lap-3$^{.90}$

"Leucine" aminopeptidase

Zones of activity for "leucine" aminopeptidase (LAP) are similar on gels of *Schizoporella unicornis* and *Bugula stolonifera*. Both species possess three loci controlling LAP, two monomorphic and one polymorphic (Table II). The enzymes of the Lap-1 loci of both species are similar in mobility (Lap-1$^{.31}$ in

TABLE III

Amount of polymorphism in esterases, malate dehydrogenase, and "leucine" aminopeptidase for Schizoporella unicornis *and* Bugula stolonifera

	Schizoporella unicornis	*Bugula stolonifera*	Totals	Per cent polymorphism
Esterases				
Monomorphic	3	2	5	
Polymorphic	1	4	5	50.0
Malate dehydrogenase				
Monomorphic	1	1	2	
Polymorphic	0	1	1	33.3
"Leucine" aminopeptidase				
Monomorphic	2	2	4	
Polymorphic	1	1	2	33.3
Totals	6 2	5 6	11 8	42.1
Per cent polymorphism	25.0	54.5	42.1	

S. unicornis and Lap-1$^{.2S}$ in *B. stolonifera*). Lap-2 enzymes are quite different in mobilities and staining properties. The Lap-3 locus in *S. unicornis* has two alleles and the corresponding locus of *B. stolonifera* has three. Once again, the protein mobilities are similar (mean mobility of 0.96 in *S. unicornis* and 0.88 in *B. stolonifera*). The similarity in banding pattern of Lap-1 and Lap-3 may indicate the presence of homologous proteins that are conservative to changes in electrophoretic mobility.

Gene and genotype frequencies and polymorphism

Genotype frequencies have been determined for 5 separate polymorphic loci (Table IV). Data for Lap-3 of *Schizoporella unicornis* are given for 3 localities making a total of 7 determinations. Gene frequencies were calculated from genotype frequencies by the formulas: $p = P + \frac{1}{2}H$, and $q = Q + \frac{1}{2}H$, where p and q

TABLE IV

Summary of gene and genotype frequencies and chi-square agreement with Hardy-Weinberg equilibrium values for Schizoporella unicornis *and* Bugula stolonifera

Species	Locality	Sample size	Locus	Gene frequency		Genotype frequency			Chi-Square agreement of genotype frequency with Hardy-Weinberg
				Low mobility allele	High mobility allele	Low mobility homozygotes	Heterozygotes	High mobility homozygotes	
Schizoporella unicornis	MBL Dock	50	Lap-3	0.720	0.280	25	22	3	no significant difference
	Green Pond	43	Lap-3	0.756	0.244	25	15	3	no significant difference
	Vineyard Sound	33	Lap-3	0.485	0.515	9	14	10	no significant difference
Bugula stolonifera	Eel Pond	44	E-2	0.864	0.136	34	8	2	no significant difference
	Eel Pond	43	E-3	0.602	0.398	20	12	11	$P < 0.01$
	Eel Pond	44	E-4	0.955	0.045	40	4	0	no significant difference
	Eel Pond	44	E-5	0.693	0.307	20	21	3	no significant difference

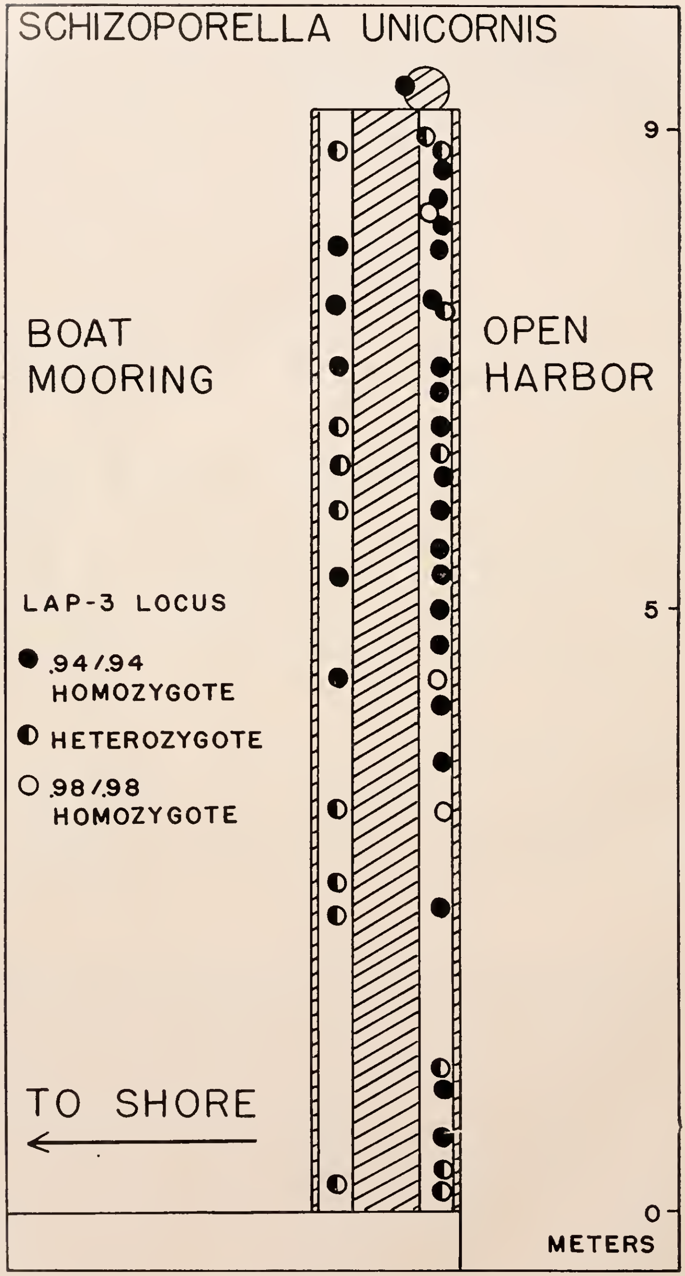


FIGURE 4. Distribution of genotypes of "leucine" aminopeptidase (locus Lap-3) in colonies of *Schizoporella unicornis* occurring on outer floating dock of boat moorings at Green Pond, Massachusetts. White area represents the surface available for colonization. Note that the boat mooring side has a greater frequency of heterozygotes than the open water side. The difference is significant at the 5 per cent level.

are gene frequencies and P, H and Q are the frequencies of homozygote, heterozygote, and other homozygote classes respectively (Falconer, 1960). The Hardy-Weinberg equilibrium distribution of genotypes was calculated from gene frequencies for the 5 loci. Genotypes obtained were compared to expected values for goodness-of-fit (Table IV). Only the genotype frequency of the E-3 locus in *Bugula stolonifera* differs significantly from Hardy-Weinberg equilibrium values. At this locus there is a significant deficiency of heterozygotes ($P < 0.01$).

In *Schizoporella unicornis* 2 of 8 or 25.0% of loci are polymorphic and in *Bugula stolonifera* 6 of 11 or 54.5% of loci are polymorphic. Mean polymorphism is 42.1% (Table III). Combined data from both species show 5 esterase loci (50%), 1 MDH locus (33.3%), and 2 LAP loci (33.3%) to be polymorphic.

Geographic variation in gene and genotype frequencies

Local populations or demes of *Bugula stolonifera* in Eel Pond extend over areas of at least 3.6 m². Genotypes of the E-5 locus are randomly distributed over this area (Fig. 3).

Collections were made from the floating dock at Green Pond and the pilings at the MBL dock which show the distribution of Lap-3 genotypes of *Schizoporella unicornis* over areas of 11–18 m² (Figs. 4, 5). At Green Pond (Fig. 4) there is a possible bias of heterozygotes ($\chi^2 = 4.26$, $P < 0.05$) on the side of the dock which is part of a motor boat mooring. The water on this side is more polluted than that of the free harbor. In contrast, the distribution of genotypes in a sample collected from several pilings of the MBL dock appears to be random (Fig. 5). The depth distribution on the pilings, however, does suggest a non-random distribution of genotypes (Fig. 5). The homozygote Lap-$3^{.94/.94}$ is absent in 22 near surface samples, occurs in 1 of 14 samples of intermediate depth, but accounts for 2 of the 4 deepest samples.

A third geographic comparison is available for populations separated on the order of kilometers (Fig. 6). *S. unicornis* from Vineyard Sound dredgings differs significantly from the MBL dock and Green Pond populations in gene frequency at the Lap-3 locus ($\chi^2 = 9.3$, $P < 0.01$, in a comparison of the MBL dock and Vineyard Sound populations). The frequency of Lap-$3^{.98}$ averages about 24% higher in the Vineyard Sound collection. Samples of MBL dock and Green Pond populations do not show significant differences.

DISCUSSION

The most important conclusion we can make is that the genetic structure of an ectoproct population appears to be fundamentally like that of genetically well-known terrestrial animals, such as *Drosophila*. This conclusion is, perhaps, surprising, because ectoprocts are colonial, sessile, and for the most part, hermaphroditic. The peculiarities of the ectoproct way of life might be expected to result in unusual sexual and genetic mechanisms. Such mechanisms were not found.

Breeding structure of local populations

Local populations of *Schizoporella unicornis* at Green Pond and MBL dock, and *Bugula stolonifera* at Eel Pond are composed of several hundred colonies.

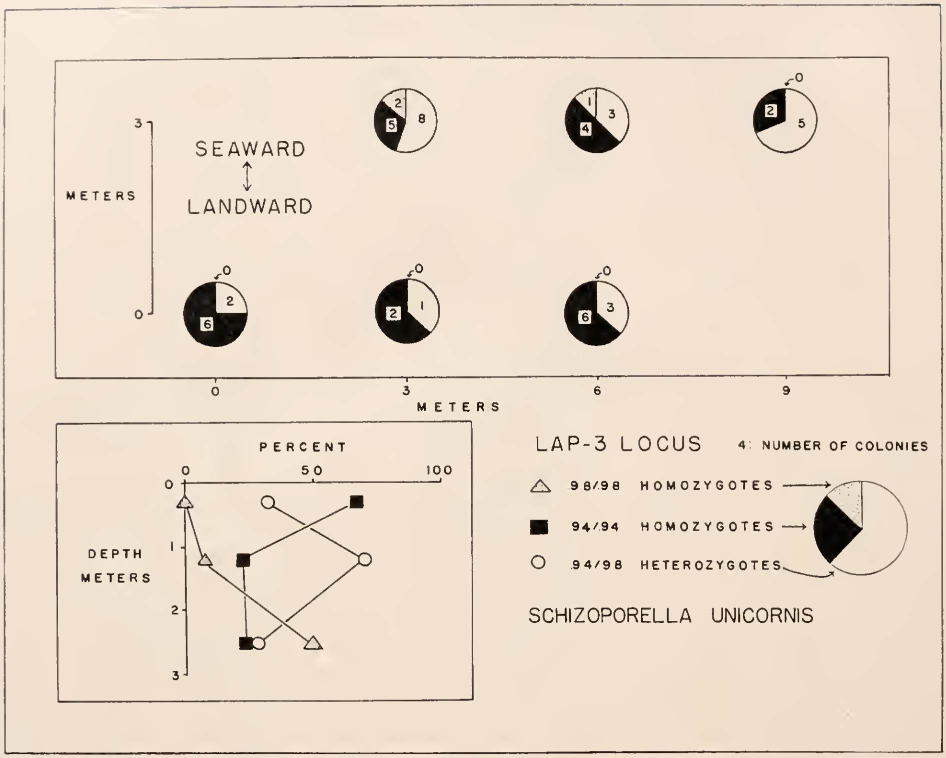


FIGURE 5. Plan view and depth distribution of genotypes of "leucine" aminopeptidase (Locus Lap-3) in colonies of *Schizoporella unicornis* occurring on "islands" of pilings at the MBL dock. In plan view, samples are summed over all depth intervals. Genotypes appear to be randomly distributed, taking into account the small sample size (3–15 colonies) on each set of pilings. In depth, samples are summed over all areas, and by depth interval (0.0–0.6 m, n = 22; 0.6–1.8 m, n = 14; 1.8–3.0 m, n = 4).

Conspicuous aggregations of like genotypes are usually not found, indicating that larvae do not habitually settle in the immediate vicinity of the parental colony. Approximately random distribution of genotypes is the rule. Apparently the sessile mode of adult existence is not a barrier to dispersal of ectoprocts over distances of square meters or tens of square meters.

Genotype frequencies at several loci in these local populations are largely in accord with Hardy-Weinberg equilibrium frequencies. We infer that individuals of each colony freely interbreed with those of other colonies in the local population. Since sperm and eggs are produced by the colonies of these species at the same time, a self-incompatibility mechanism may be present to prevent intra-colony fertilization. Nevertheless, some inbreeding may exist in ectoproct populations, but has escaped detection. As an example, the sample of 50 colonies of *Schizoporella unicornis* from the MBL dock is too small to detect a coefficient of inbreeding (F) of 0.25 at the 0.5 level of significance of the chi-square test.

The close agreement with Hardy-Weinberg genotype frequencies suggests that alleles at the loci sampled are selectively neutral. However, Lewontin and Cockerham (1958) have shown that statistical agreement with Hardy-Weinberg values can be an insensitive indicator of selection in small population samples. It is distinctly possible that selection pressures of 10–20% are operating against particular genotypes. There is tentative evidence for this viewpoint since genotypes of the Lap-3 locus in *S. unicornis* depart from random distribution along environmental gradients. At Green Pond a positive bias of heterozygotes appears to occur on the side of the floating dock closer to a source of oil and gasoline pollution (Fig. 4). At the MBL dock, the Lap-$3^{.94/.94}$ homozygotes become proportionately more abundant with increasing depth (Fig. 5).

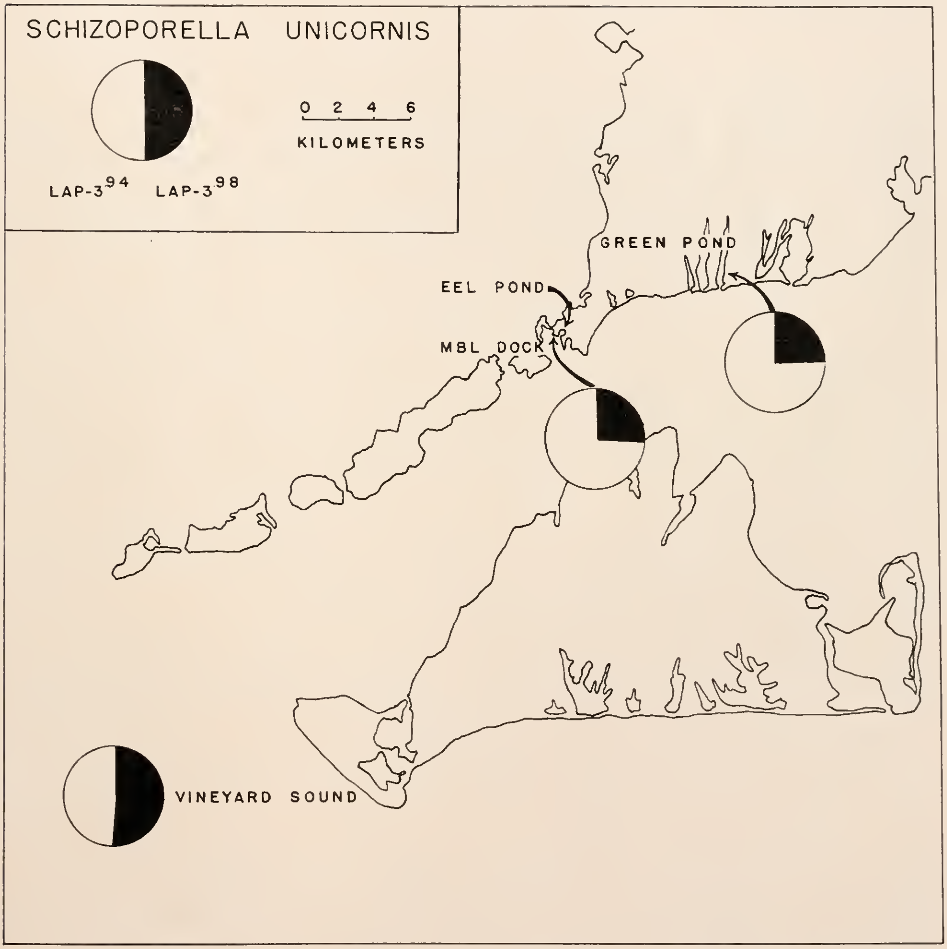


FIGURE 6. Map showing location of collecting localities from Green Pond, MBL dock, and Vineyard Sound dredging, together with gene frequencies for Lap-3 alleles. Note that the coastal samples have nearly identical gene frequencies whereas the Vineyard Sound frequency is distinctly different.

The only locus to show statistically significant departure from Hardy-Weinberg equilibrium values ($P < 0.01$) is the E-3 locus in Eel Pond populations of *B. stolonifera*. There is a marked deficiency of the heterozygote class. Since genotypes at the other sampled loci conform to equilibrium frequencies, self-fertilization or close inbreeding are not responsible. Inbreeding would lead to a deficiency of heterozygotes at all loci. Positive assortive fertilization could account for the deficit of heterozygotes, by which gametes carrying either the $E\text{-}3^{.90}$ or $E\text{-}3^{.94}$ allele preferentially fertilize eggs bearing the like allele. Alternatively, fertilization may be random but heterozygote mortality is greater. Either explanation requires linkage equilibria between E-3 and the other loci sampled. This problem cannot be resolved until more is known of gamete behavior and the relative fitness of E-3 genotypes.

Genetic variability of local populations

The genome of an ectoproct probably contains thousands of loci. Our data concerning genetic variability in ectoprocts are based on 8 loci in *S. unicornis* and 11 loci in *B. stolonifera*, which are small samples. Other limitations of electrophoretic methods in the determinations of genetic variability are discussed in Shaw (1965), Lewontin and Hubby (1966), and O'Brien and MacIntyre (1969). The most important biases recognized by these authors which are applicable to our research are:

(1) A genetic variant that does not alter the mobility of its protein product will not be detected.

(2) Alleles occurring at low frequencies in populations may not be sampled unless large numbers of individuals are analyzed.

(3) Different mutations of the same gene may cause an identical alteration in mobility of a polypeptide. Thus two genetic variants may be recorded as a single electrophoretic variant.

(4) Histochemical methods are known for a relatively small number of proteins, most of which are enzymes. The amount of polymorphism in genes whose products are other classes of protein is unknown.

Most biases inherent in electrophoresis methodology tend to underestimate the amount of genetic variability. Limitations (1), (2), and (3) are of this type. The limitations in methodology are not likely to cause recent estimates of genetic variability, including those reported here, to be drastically revised downward.

Our analysis of genetic variability in ectoprocts yields estimates consistent with those recently made for man and several species of *Drosophila* (summarized by O'Brien and MacIntyre, 1969). The percentage of polymorphic loci in two ectoproct species is 25.0 and 54.5%, with a mean of 42.1%. If we add unpublished data for 8 loci each of two other species of *Bugula*, the mean value becomes 48.5%. The range of estimates of loci in *Homo sapiens* and *Drosophila* is 30.0–54.0%, with a mean of 42.0%, disregarding a single estimate of 100% based on only 3 loci. The average proportion of the genome heterozygous per individual (calculated after Lewontin and Hubby, 1966) is 0.07 in *S. unicornis* and 0.21 in *B. stolonifera* compared with a range of 0.12–0.33 obtained for man and *Drosophila*.

If these estimates of the amount of polymorphism are typical of the terrestrial and marine taxa they represent, then little seems to distinguish the colonial, sessile, and hermaphroditic marine ectoprocts from solitary, free living, unisexual terrestrial animals in the genetic variability maintained in populations.

Consequences of spatial separation

The marine realm is frequently stated to be more homogenous and stable than the terrestrial (David, 1963) with the notable exception of the highly-variable intertidal environment (Knox, 1963). Some scientists have regarded the apparent scarcity of barriers to migration in the sea as evidence of sympatric speciation (Kohn, 1960; Weiser, 1960; and Day, 1963). Ernst Mayr, by no means an advocate of sympatric speciation, considered that free gene flow has led to widespread panmictic conditions in marine animals as opposed to local genetic differentiation (Mayr, 1963).

Two contrasting models of marine ectoproct populations may be proposed. According to the one just cited, extensive gene flow among populations of marine ectoprocts would militate against the formation of semi-isolated local populations. Species would be cosmopolitan aggregations of individuals and major shifts in gene frequencies would only occur over extensive areas differing considerably in important environmental parameters such as temperature and salinity.

The other model calls for a marine environment beset (from an ectoproct point of view) with barriers to gene flow. Micro-environmental changes in substrate relief, texture, and composition, current strength and direction, quality and quantity of nutrients, and salinity and temperature might serve to disrupt genetic communication between local populations. This model would permit divergence in gene frequencies due either to responses to local selection pressures or, in small populations, to genetic drift, or a combination of selection and drift. Small, semi-isolated populations are commonly found in terrestrial animals such as the snail *Cepaea nemoralis* (Cain and Sheppard, 1954), species of *Drosophila* (Wallace, 1968), and certain human populations (Cavalli-Sforza, 1959).

Our evidence is insufficient to fully evaluate these models, but it is more consistent with a model of small, semi-isolated populations than with the concept of widespread panmixia in the sea. An approximate 24 per cent disparity exists in the frequency of alleles of the Lap-3 locus in *S. unicornis* between the Vineyard Sound population and the near-shore Green Pond and MBL dock populations. Whether the allele frequencies change clinally or abruptly through the intervening populations is not yet known. In either case the 35 km interval serves as a partial barrier to gene flow or else stringent differential selection maintains the difference in the face of gene flow. Either the existence of effective barriers to gene flow or strong local selection pressures would disrupt panmixia among populations of marine species.

More work needs to be done to assess the potentialities of dispersal in ectoprocts. The larvae of *S. unicornis* are planktonic and therefore subject to rapid current dispersal. However, the larvae are brooded for several days and are released into the water column for a free life of only hours. They have no digestive tract and are nourished by a yolk supply. Estimates of current pattern and strength (Sumner, Osburn, Cole and Davis, 1913; Coast and Geodetic Survey tidal current charts, 1949) show it requires more than one day for water at the MBL dock to be transported to the Vineyard Sound location assuming a direct and rapid rate of transport. Thus, biological and physical limitations to rapid dispersal appear to be operative. Such gene flow as does occur between these populations must be via intervening populations.

All of the work reported here was conducted at the Marine Biological Laboratory, Woods Hole, the use of whose facilities we gratefully acknowledge. Technical assistance was given by Mr. K. W. Kaufmann and Mrs. E. Gooch. Supported in part by NSF grant GB-7325 to Thomas J. M. Schopf.

SUMMARY

Proteins of marine species of the Phylum Ectoprocta can be easily separated by zone electrophoresis on polyacrylamide gels. Many bands revealed by electrophoresis represent the product of individual genetic loci from which gene and genotype frequencies were calculated. Results are reported for 19 loci which are responsible for the formation of esterases, malate dehydrogenase and "leucine" aminopeptidase in *Schizoporella unicornis* and *Bugula stolonifera*.

Observed genotype frequencies correspond closely with those predicted by Hardy-Weinberg equilibria indicating that these marine ectoprocts are dominantly outbreeding and that panmictic local populations cover an area of square meters. In addition, 25.0–54.5% of the diagnosed loci are polymorphic, depending on the species. Forty-two % of all loci identified are polymorphic.

The most important implication of these findings is that the population structure and amount of genetic variability in ectoprocts (and by implication in other marine organisms) is fundamentally similar to that of terrestrial organisms. If these tentative conclusions are substantiated by subsequent work, the processes of speciation in marine organisms may differ little from those proposed in the better documented research on terrestrial animals.

LITERATURE CITED

ALLEN, S. L., 1961. Genetic control of the esterases in the protozoan *Tetrahymena pyriformis*. *Ann. New York Acad. Sci.*, **94**: 753–773.

AR-RUSHDI, A. H., 1958. The polygenic basis of sex-ratio in *Tigriopus*. *Proc. 10th Int. Congr. Genet.*, **2**: 8–9.

AUGUSTINSSON, K-B., 1961. Multiple forms of esterase in vertebrate blood plasma. *Ann. New York Acad. Sci.*, **94**: 844–860.

BARIGOZZI, C., 1960. Genetic systems in sessile and semisessile organisms, pp. 559–569. *In:* A. A. Buzzati-Traverso, Ed., *Perspectives in Marine Biology*. University of California Press, Berkeley.

BATTAGLIA, B., 1958. Balanced polymorphism in *Tisbe reticulata*, a marine copepod. *Evolution*, **12**: 358–364.

BATTAGLIA, B., 1965. Advances and problems of ecological genetics in marine animals. *Proc. 11th Int. Congr. Genet.*, **2**: 451–463.

BECKMAN, L., AND F. M. JOHNSON, 1964a. Esterase variations in *Drosophila melanogaster*. *Hereditas*, **51**: 212–220.

BECKMAN, L., AND F. M. JOHNSON, 1964b. Genetic control of aminopeptidases in *Drosophila melanogaster*. *Hereditas*, **51**: 221–230.

BOCQUET, C., 1951. Recherches sur *Tisbe* (*-Idyea*) *reticulata*, n. sp. *Arch. Zool. Exp. Gén.*, **87**: 335–416.

Bocquet, C., 1953. Recherches sur le polymorphisme naturel des *Jaera marina* (Fabr.) (Isopodes Asellotes). *Arch. Zool. Exp. Gén.*, **90**: 187–450.

Bullivant, J. S., 1967. Release of sperm by Bryozoa. *Ophelia*, **4**: 139–142.

Burns, J. M., and F. M. Johnson, 1967. Esterase polymorphism in natural populations of a sulfur butterfly *Colias eurytheme*. *Science*, **156**: 93–96.

Buzzati-Traverso, A. A., 1960. Perspectives in marine biology, pp. 613–621. *In:* A. A. Buzzati-Traverso, Ed., *Perspectives in Marine Biology*. University of California Press, Berkeley.

Cavalli-Sforza, L. L., 1959. Some data on the genetic structure of human populations. *Proc. 10th Int. Congr. Genet.*, **1**: 389–407.

Cain, A. J., and P. M. Sheppard, 1954. The theory of adaptive polymorphism. *Amer. Natur.*, **88**: 321–326.

Coast and Geodetic Survey, 1949. Tidal current charts: Narragansett Bay to Nantucket Sound, second edition. *Serial No. 628*. U. S. Dept. Commerce, 12 charts.

David, P. M., 1963. Some aspects of speciation in the Chaetognatha, pp. 129–143. *In:* J. P. Harding and N. Tebble, Eds., *Speciation in the Sea*. The Systematics Association, London.

Day, J. H., 1963. The complexity of the biotic environment, pp. 31–49. *In:* J. P. Harding and N. Tebble, Eds., *Speciation in the Sea*. The Systematics Association, London.

Falconer, D. S., 1960. *Introduction to Quantitative Genetics*. Ronald Press Co., New York, 365 pp.

Føyn, B. and I. Gjøen, 1954. Studies on Serpulid *Pomotoceros triqueter* L. II. The colour pattern of the branchial crown and its inheritance. *Nytt Mag. Zool.*, **2**: 85–90.

Gold, A. M., 1967. Sulfonylation with sulfonyl halides, pp. 706–711. *In:* C. H. W. Hirs, Ed., *Methods in Enzymology, Volume 11,* Enzyme structure. Academic Press, New York.

Hubby, J. L., 1963. Protein differences in *Drosophila*. I. *Drosophila melanogaster*. *Genetics*, **48**: 871–879.

Hubby, J. L., and R. C. Lewontin, 1966. A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at different loci in *Drosophila pseudoobscura*. *Genetics*, **54**: 577–594.

Hyman, L. H., 1959. *The Invertebrates, Volume 5*. McGraw-Hill Book Co., New York, 783 pp.

Jacobson, K. B., 1968. Alcohol dehydrogenase of *Drosophila:* interconversion of isoenzymes. *Science*, **159**: 324–325.

Johnson, F. M., C. G. Kanapi, R. H. Richardson, M. R. Wheeler and W. S. Stone, 1966. An analysis of polymorphisms among isozyme loci in dark and light *Drosophila ananassae* strains from American and Western Samoa. *Proc. Nat. Acad. Sci. U. S. A.*, **56**: 119–125.

Knox, G. A., 1963. Problems of speciation in intertidal animals with special reference to New Zealand shores, pp. 7–29. *In:* J. P. Harding and N. Tebble, Eds., *Speciation in the Sea*. The Systematics Association, London.

Kohn, A. J., 1960. Problems of speciation in marine invertebrates, pp. 571–588. *In:* A. A. Buzzati-Traverso, Ed., *Perspectives in Marine Biology*. University of California Press, Berkeley.

Kojima, K., and K. M. Yarbrough, 1967. Frequency dependent selection at the *Esterase-6* locus in *Drosophila melanogaster*. *Proc. Nat. Acad. Sci. U. S. A.*, **57**: 645–649.

Lewontin, R. C., and C. C. Cockerham, 1958. The goodness-of-fit test for detecting natural selection in random mating populations. *Evolution*, **13**: 561–564.

Lewontin, R. C., and J. L. Hubby, 1966. A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics*, **54**: 595–609.

Makino, S., 1951. *An Atlas of the Chromosome Numbers in Animals*. Iowa State College Press, Ames, Iowa, 240 pp.

Manwell, C., 1966. Sea cucumber sibling species: Polypeptide chain types and oxygen equilibrium of hemoglobin. *Science*, **152**: 1393–1396.

Manwell, C., and C. M. A. Baker, 1963. A sibling species of sea cucumber discovered by starch gel electrophoresis. *Comp. Biochem. Physiol.*, **10**: 39–53.

Manwell, C., and C. M. A. Baker, 1968. Genetic variation of *iso*citrate, malate and 6-phosphogluconate dehydrogenases in snails of the genus *Cepaea*—introgressive hybridization, polymorphism and pollution? *Comp. Biochem. Physiol.,* **26**: 195–209.

Manwell, C., C. M. A. Baker, P. A. Ashton and E. D. S. Corner, 1967. Biochemical differences between *Calanus finmarchicus* and *C. helgolandicus. J. Mar. Biol. Ass. U. K.,* **47**: 145–169.

Maturo, F. J. S., Jr., 1966. Bryozoa of the southeast coast of the United States: Bugulidae and Beaniidae (Cheilostomata: Anasca). *Bull. Mar. Sci.,* **16**: 556–583.

Mayr, E., 1963. *Animal Species and Evolution.* Belknap Press of Harvard University Press, Cambridge, Massachusetts, 797 pp.

Milkman, R., 1967. Genetic and developmental studies on *Botryllus schlosseri. Biol. Bull.,* **132**: 229–243.

Moore, R. O., and C. A. Villee, 1963. Multiple molecular forms of malic dehydrogenase in echinoderm embryos. *Comp. Biochem. Physiol.,* **9**: 81–94.

O'Brien, S. J., and R. J. MacIntyre, 1969. An analysis of gene-enzyme variability in natural populations of *Drosophila melanogaster* and *D. simulans. Amer. Natur.,* **103**: 97–113.

Ogita, Z., and T. Kasai, 1965. Genetico-biochemical analysis of specific esterases in *Musca domestica. Jap. J. Genet.,* **40**: 173–184.

Ray, D. L., 1960. Some marine invertebrates useful for genetic research, pp. 497–512. *In:* A. A. Buzzati-Traverso, Ed., *Perspectives in Marine Biology.* University of California Press, Berkeley.

Ryland, J. S., 1960. The British species of *Bugula* (Polyzoa). *Proc. Zool. Soc. London,* **134**: 65–105.

Ryland, J. S., 1965. *Catalogue of Main Marine Fouling Organisms. Vol. 2: Polyzoa.* Organization for Economic Co-operation and Development, Paris, 83 pp.

Sabaddin, A., 1959. Analisi genetica del policromatismo di *Botryllus schlosseri* (Pallas) Savigny (Ascidiacea). *Boll. Zool.,* **26**: 221–243.

Scandalios, J. G., 1969. Genetic control of multiple molecular forms of enzymes in plants: A review. *Biochem. Genet.,* **3**: 37–79.

Schopf, T. J. M., and F. T. Manheim, 1967. Chemical composition of Ectoprocta (Bryozoa). *J. Paleontol.,* **31**: 1197–1225.

Schopf, T. J. M., and F. T. Manheim, 1968. Chemical composition of Ectoprocta (Bryozoa): Addenda. *J. Paleontol.,* **42**: 858.

Sexton, E. W., and A. R. Clark, 1936. Summary of the work on the amphipod *Gammarus chevreuxi* Sexton carried out at the Plymouth Laboratory (1912–1936). *J. Mar. Biol. Ass. U. K.,* **21**: 357–414.

Shaw, C. R., 1965. Electrophoretic variation in enzymes. *Science,* **149**: 936–943.

Silén, L., 1966. On the fertilization problem in the gymnolaematous Bryozoa. *Ophelia,* **13**: 113–140.

Staiger, H., 1954. Der Chromosomendimorphismus beim Prosobranchier *Purpura lapillus* in Beziehung zur Ökologie der Art. *Chromosoma,* **6**: 419–478.

Stone, W. S., M. W. Wheeler, F. M. Johnson and K. Kojima, 1968. Genetic variation in natural island populations of *Drosophila nasuta* and *Drosophila ananassae* subgroups. *Proc. Nat. Acad. Sci. U. S. A.,* **59**: 102–109.

Sumner, F. B., R. C. Osburn, L. J. Cole and B. M. Davis, 1913. A Biological Survey of the Waters of Woods Hole and Vicinity. *Bull. Bur. Fish. U. S.,* **31** (2 pts): 1–861.

Thornber, E. J., I. T. Oliver and P. B. Scutt, 1968. Comparative electrophoretic patterns of dehydrogenases in different species. *Comp. Biochem. Physiol.,* **25**: 973–987.

Wallace, B., 1968. *Topics in Population Genetics.* W. W. Norton and Co., Inc., New York, 481 pp.

Wieser, W., 1960. Problems of species formation in benthic microfauna of the deep-sea, pp. 513–518. *In:* A. A. Buzzati-Traverso, Ed., *Perspectives in Marine Biology.* University of California Press, Berkeley.

Wright, T. R. F., 1963. The genetics of an esterase in *Drosophila melanogaster. Genetics,* **18**: 787–801.

Yarbrough, K., and K. Kojima, 1967. The mode of selection at the polymorphic Esterase-6 locus in cage populations of *Drosophila melanogaster. Genetics,* **57**: 677–686.

Reference: *Biol. Bull.,* **138**: 157–183. (April, 1970)

OSMOTIC AND IONIC REGULATION IN THE HORSESHOE CRAB *LIMULUS POLYPHEMUS* (LINNAEUS)

JAMES D. ROBERTSON

Zoology Department, University of Glasgow, Glasgow, Scotland, U. K. and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Limulus polyphemus is one of the four living species of horseshoe crabs, a group with a geological history which can be traced back to the Silurian (*Neolimulus*) and even to the Cambrian (Størmer, 1952). The Mesozoic species originally referred to the genus *Limulus* are closely related and similar to present-day forms and *Mesolimulus walchi* of the Jurassic Solnhofen lagoons co-existed with a fauna which included jelly-fishes, swimming crinoids and ammonites (Abel, 1927). During the intervening 135 million years (Howarth, 1964) the xiphosurans of the lagoons and seas covering parts of Germany, France, England, Sweden and N. Africa have disappeared leaving three living genera, one on the east coast of North America (*Limulus*), and the other two on the south-east and east coasts of Asia (*Tachypleus* and *Carcinoscorpius*) (Størmer, 1952).

The American *Limulus polyphemus* is found in coastal and estuarine areas from Nova Scotia to Yucatan, the adults migrating shorewards in spring and summer to spawn intertidally on sandy beaches (Shuster, 1957, 1960). Probably all stages tolerate a moderate reduction in salinity and known populations of *Limulus* occur in areas where there are considerable ranges of salinity, *e.g.*, Miles River, Maryland (salinity 8–17‰), Ashepoo River and St. Helena Sound, South Carolina (18.4–30.6‰), Hales Cove, Plum Island Sound, Massachusetts (6–32‰) (Shuster, 1966, 1969 personal communication), Delaware Bay, New Jersey (7–30‰) (McMannus, 1969). However, in Barnstable Harbor, on the north shore of Cape Cod, Massachusetts where nests of eggs are to be found in early summer at mid-tide level (Shuster, 1950), the poor run off is slight in relation to the sea water entering and leaving the embayment, resulting in an essentially marine aquatic environment for all stages of *Limulus*. During 1947–1948 the salinity was 29–32‰ at both low and high tides, exceptionally 20–31‰ during spring freshet conditions (Ayers, 1959).

In normal sea water there is dispute about whether the animal is isosmotic (Garrey, 1905; Dailey, Fremont-Smith and Carroll, 1931) or hyperosmotic (Cole, 1940; and Homer W. Smith, in Cole, 1940). McManus (1969) states that *Limulus* is approximately isosmotic in water of about 25‰ salinity, and finds that large specimens (prosomal width 15 cm or more) in water of about 7‰ were slightly hyperosmotic after 24 hours. Twelve out of twenty young crabs (prosomal width 7.5 cm or less) survived a salinity of 2.5‰ for 127 hours. Longer term results at Woods Hole by Pearse (1928) have shown that small individuals (<10 cm long) survive for 2–3 weeks in 25% sea water (about 8‰), dying in weaker solutions (4‰) within 2 days.

The present work was started at Woods Hole in the summer of 1966, with the object of investigating the salinity tolerance and powers of osmotic and ionic regulation of the horseshoe crab. It was extended to include determinations of the principal osmotic constituents of muscle at different levels of external salinity. Some data on the latter aspect have been published by Bricteux-Grégoire, Duchâteau-Bosson, Jeuniaux and Florkin (1966).

MATERIAL AND METHODS

Specimens were obtained from the M. B. L. holding tanks at Woods Hole, and kept in the laboratory in large tanks of gently flowing sea water at 21–23° C. During the period 25th July to 1st September, 1966, the chloride content of the sea water varied only slightly 18.01–18.15 g/liter, with a mean value of 18.07. Corresponding chlorinity values were 17.62–17.76 g/kg, which are equivalent to a salinity of 31.83–32.09 g/kg, the mean being 31.94. For experiments on the effect of salinity changes, specimens were kept individually in well-aerated sea water in plastic containers, dilutions of the water being made with tap-water (a soft water containing about 11 mg chloride per liter), and higher concentrations being obtained with artificial sea water double the normal Woods Hole concentration.

Animals used were chiefly those of carapace (prosoma) width 9.0–9.7 cm and 83–118 g, also several of about 7 cm width, 32–36 g. Large male adults of 17.9–19 cm width, weighing about 620–700 g, were taken for the comparative analyses of blood and muscle. The appearance of the genital apertures was used to sex the animals (Lochhead, 1950). They were not fed during the experiments or during the period, some days to a few weeks, when they were kept in the holding tanks after being trawled.

Blood was withdrawn by syringe or by pipette after a small incision had been made in the ligament joining prosoma and opisthosoma, and then the serum was separated by centrifugation after aggregation of the cells. Removal of the dorsal exoskeleton in large specimens required the use of an old-fashioned can opener (Lochhead, 1950), before the muscles could be obtained. Those used for analysis were the intertergal muscle and the flexors and extensors of the opisthosoma and telson. After they had been blotted lightly with filter paper, separate samples were taken for the determination of water and inorganic cations, and for making trichloracetic acid extracts, tungstic acid extracts and zinc hydroxide extracts.

Methods for sodium, potassium, calcium, chloride and sulphate were those described previously (Robertson, 1949, 1960a), for magnesium that of Heagy (1948). Procedures used for α-amino nitrogen, proline, trimethylamine oxide, lactate, phosphate, and copper are listed in Robertson (1961). Microdiffusion methods of Conway (1962) were used for ammonium ions of muscle and for the final determination of total non-protein nitrogen after micro-Kjeldahl digestion of samples of the tungstic acid filtrates of muscle. Arginine was estimated in trichloracetic acid filtrates by the methods of Janus (1956) and of Ennor (1957), with good agreement between the two.

Extracellular volume of specimens and of the muscle was determined by injecting into the haemocoele a measured volume of a 10% solution of inulin (Kerfut) in sea water (1 ml for specimens of 100–118 g, 5 ml for a specimen of 634 g). After periods varying from 5 to 10.3 hours in different animals, samples of blood and muscle were removed, zinc hydroxide filtrates prepared, and their content of inulin measured by the procedure of Roe, Epstein and Goldstein (1949). Chloride was estimated in the same filtrates, so that both chloride "spaces" and presumed extracellular (inulin) spaces could be compared.

Water contents were obtained by measuring the differences between fresh and dry weights; plasma samples were dried for 4 hours and muscle for 24 hours at 100–101° C. Animals were weighed to the nearest 0.1 g when the effect of changed external salinities was being investigated.

Measurements of osmotic concentration of blood serum were made thermoelectrically with the Krogh-Baldes thermocouple (Krogh, 1939). Standard deviation of 13 estimations on sodium chloride solutions of approximately the same vapor pressure as sea water was 0.56%.

From the vapor pressure and osmotic equivalence measurements of Robinson (1954) and the osmotic coefficients of sodium chloride from Robinson and Stokes (1959), sea waters of concentrations Cl 10‰ (≡ 0.2821 *M*-NaCl), Cl 18‰ (≡ 0.5263 *M*-NaCl) and Cl 22‰ (≡ 0.6503) have the following concentrations in milliosmoles: 528, 970 and 1202. From the freezing-point data of Scatchard and Prentiss (1933) and assuming solutions of sodium chloride and sea water of the same vapor pressure at 25° C have the same freezing points, then the three sea waters would freeze at −0.974° C, −1.780° C and −2.198° C and have the following concentrations (Dick, 1959, Δ 1.858° C ≡ 1000 milliosmoles): 524, 958 and 1184 milliosmoles. Measurements of the osmotic concentration of blood serum and sea water, obtained first as concentrations in relation to the standard drops (3.000 g and 1.000 g NaCl in 100 g water, the one closest to the anticipated concentration being used), were converted to milliosmoles by use of the appropriate osmotic coefficients. Some values for sea water were obtained from the first set of equivalencies above.

Osmotic regulation

(1) *Osmotic concentration and chloride values of blood-serum in relation to external medium.* In Table I are given the results of vapor pressure and chloride analyses of *Limulus* serum from specimens kept in dilutions and concentrations of sea water from 5–200% for periods of 2–15 days, as well as from normal sea water. The results show that in 66% sea water and below *Limulus* osmoregulates, maintaining higher osmotic and chloride concentrations in the blood than in the sea water. Only small *Limulus* could stand the lowest dilution of 5% sea water, those of 32–36 g and about 7 cm carapace width, and then only if acclimatized at intermediate salinities. At this dilution, osmotic and chloride concentrations of the serum were 4–6 times those of the sea water.

In normal sea water *Limulus* is isosmotic, and the values show no significant difference in relation to sex. Taking the ten specimens (♂♂ and ♀♀) together, the mean milliosmolar values of the serum are 955 (S.D. 4.1) compared with 951 for sea water, the range being 949–960. Animals placed in 150% and 200% sea water remained lively and apparently perfectly healthy; they became isosmotic within 1.5% with the new medium.

A linear relationship holds if chloride values are plotted osmotic concentration. Multiplying the chloride concentration by 56.3 for measurements per milliliter

TABLE 1

Osmotic concentration and chloride values of blood-serum in relation to the external medium

Approximate strength of sea water (per cent)	Osmotic concentration			Chloride			Water	Number and sex	Duration in sea water (hours)
	Milliosmoles		Serum as per cent sea water	mg/ml.		mg/g water	mg/ml		
	Sea water	Serum		Sea water	Serum	Serum	Serum		
200	2084	2058	98.8	38.34	36.64	38.85	945	1 ♂	48
200	2006	1976	98.5	37.16	35.06	37.74	929	1 ♀	99
200	1791	1770	98.8	32.80	30.55	32.40	943	1 ♀	71
150	1382	1372	99.3	25.69	24.55	25.55	961	1 ♀	75
150	1376	1392	101.2	25.58	24.37	25.80	945	1 ♀	74
100	950 ± 0.01	954 ± 2.10	100.4 ± 0.22	18.03 ± 0.01	17.48 ± 0.13	18.45 ± 0.12	948 ± 6.8	6 ♂	—
100	951	957 ± 1.19	100.6 ± 0.12	18.04	17.52 ± 0.25	18.44 ± 0.23	950 ± 4.9	4 ♀	—
66	[630]	699	111.0	12.04	12.77	13.38	954	1 ♂	50
66	[630]	718	114.0	12.04	12.82	13.61	942	1 ♀	50
50	[483]	569 ± 41.3	117.9 ± 8.5	9.27	10.20 ± 0.76	10.91 ± 0.83	936 ± 17.0	3 ♂	50; 48 in 80% and 66%
50	[482]	638 ± 21.5	132.3 ± 4.5	9.25	11.43 ± 0.44	12.01 ± 0.45	952 ± 1.0	2 ♀	
33	[323]	476 ± 34.7	147.4 ± 10.7	6.20	8.42 ± 0.52	8.94 ± 0.58	943 ± 11.7	4 ♂	48; 50 in 50%
25	231	372 ± 44.1	161.1 ± 19.1	4.45	6.58 ± 0.90	7.02 ± 0.93	936 ± 6.4	3 ♂	48–50; 24 in 50%
20	187	419 ± 16.0	224.1 ± 8.5	3.61	7.19 ± 0.04	7.68 ± 0.20	937 ± 28.5	2 ♂	48; 50 in 50% and 33%
15	[141]	176 ± 5.0	125 ± 3.5	2.72	2.98 ± 0.12	3.26 ± 0.07	915 ± 15.0	2 ♀	113–144; 46 in 20%
10	[95]	293 ± 6.0	308 ± 6.3	1.84	5.02 ± 0.27	5.26 ± 0.16	953 ± 21.0	2 ♂	50–79; 16–50 in 50%, 33% and 20%
5	[48]	287 ± 34.5	598 ± 71.7	0.92	4.91 ± 0.91	5.07 ± 0.86	961 ± 19.4	3 ♂	40–111; 47–244 in 50%, 33%, 20% and 10%
5	[47]	229	487	0.91	4.23	4.25	996	1 ♀	

[ ] = values calculated from chloride titrations.

± = standard error.

Three of specimens in 50%, two in 33% and all those in lower salinities (except one in 15%) had periods of acclimatization at intermediate salinities, given in last column, after the semi-colon; the first figure is number of hours in the final dilution.

and by 53.2 for those per gram water give calculated values for osmotic concentration differing only slightly from those found experimentally. In both cases the average deviation from the figures in the table is under 2%, and using the 53.2 factor only two of the eighteen calculated concentrations exceed 2.9%. But in calculations of osmotic concentration from serum chloride in the ten specimens in full-strength (100%) sea water, factors of 54.6 and 51.8 are more appropriate, the average deviation from measured values being nil in both cases, with ranges of 0.2–4.1% and 0.1–2.4%, depending on whether volume or water content figures are used.

Figure 1 shows the relationship in osmotic concentration between serum and sea water, after plotting the results for each specimen (30 points, the closely

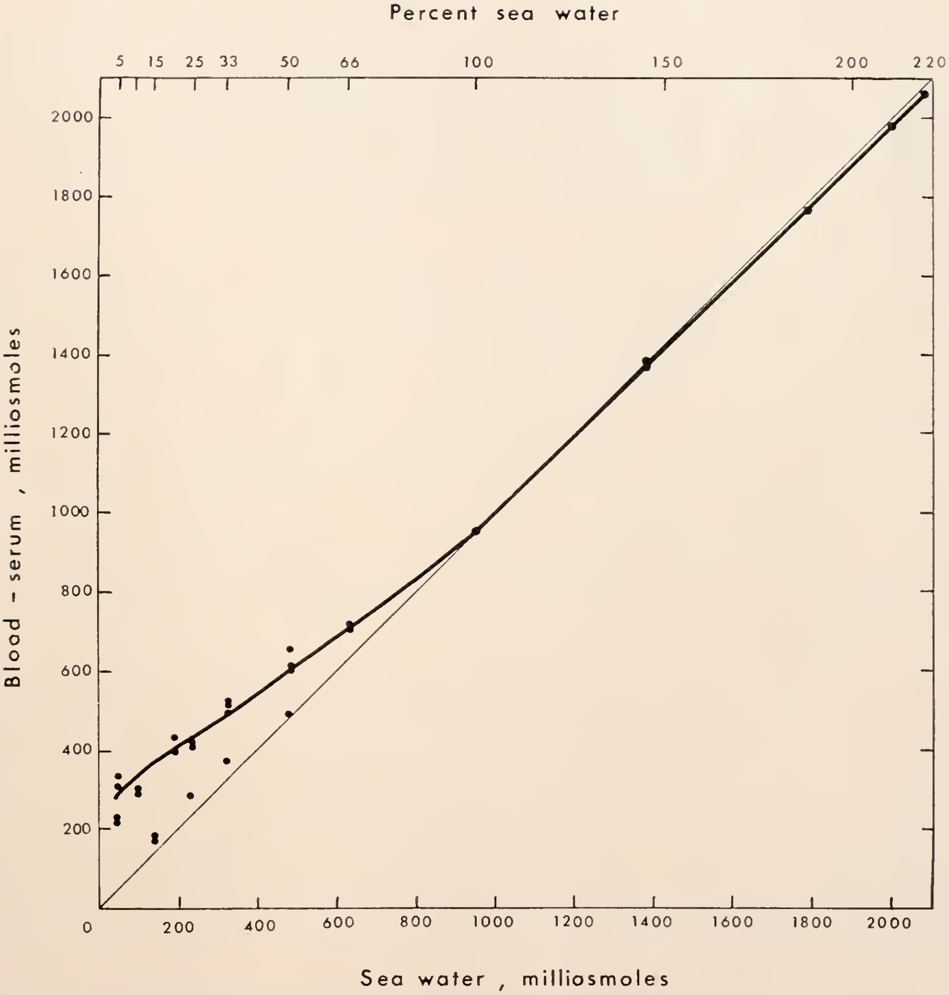


FIGURE 1. The osmotic concentration of *Limulus* blood in relation to that of the external medium.

agreeing values for the ten in 100% sea water being plotted as one point). Five points are near the isosmotic line, all animals of 109–114 g. Four of these animals had been weighed and two particularly, those in 15% sea water, showed increases to 122% and 116% of their initial weight after 6–8 days (the first having been transferred directly to 15% from 100%, the second having a preliminary 2-day period in 20%). Presumably these animals had been overstressed. Of all those in diluted sea water recorded in Figure 1, they were the only ones which did not seem perfectly healthy. When examined out of water the normal resistance to straightening of the flexed opisthosoma had somewhat decreased by the end of the experimental period. Further data on weight changes in general are given in the next section.

Taken as a whole the data in Figure 1 and Table I show that *Limulus* is a hardy animal, able to survive extensive dilutions and concentrations of sea water by maintaining (1) an osmotic steady state at concentrations of about 90–200% sea water, and (2) hyperosmotic regulation at salinities between 5 and 90%. Under experimental conditions the remarkable tolerance of the animal for at least periods of 2–5 days (15 days in the case of the smaller specimens in 5% sea water) is shown by the fact that at the lowest salinity tolerated and at the highest salinity investigated there is a seven-fold difference in blood values of total concentration and chloride.

(2) *Changes in weight and in water content of* Limulus *in different salinities.* Two *Limulus* in 100% sea water were weighed at intervals for 11–15 days. The first of 104.3 g was weighed a further five times, the mean weight being 104.9 g, with a range of 104.3–105.6 g (100–101.2%). The second of 92.4 g was weighed an additional nine times, the mean weight being 91.6 g, range 91.0–92.4 g (98.5–100%).

In Figure 2 are shown percentage changes in weight of specimens transferred directly to dilute and concentrated sea waters over a 10–200% range. Down to a concentration of 33% sea water, weight increases are small, some 2–4%, rather greater in 20% sea water, 5–8%, but quite large, 13–22%, in 15% and 10% sea water. For these medium-sized horseshoe crabs (92–118 g) salinities of 10–15% are critical levels for survival beyond a few days in such direct transfer experiments.

In concentrated sea waters weight was lost, and then regained or partially regained. The two animals in 150% sea water lost 3% of their weight in 6 hr, but regained it in 75 hr, while the three in 200% lost nearly 6% in the same time and none had fully regained original weight when taken for analysis of the blood (the two kept for the longest periods, 71 and 99 hr, attaining 98.3% and 97.6%).

The reversibility of weight increases is shown in Figure 3, where after 84 hr in dilute sea waters of 20% and 15% the weight had increased to 123.5% of the initial value. On return to full-strength sea water, a fairly rapid decrease in weight resulted, apparent in an hour, with a fall to 108.5% in 8 hr and to 100.9% in 22 hr. Thereafter a slight rise occurred to 103.9% 5 days after the return to 100% sea water.

There is a marked difference in total water content of intermolt animals in 100% sea water and in 5%, some 10% difference, which is perhaps slightly exaggerated by a presumed relatively lighter exoskeleton in the younger animals

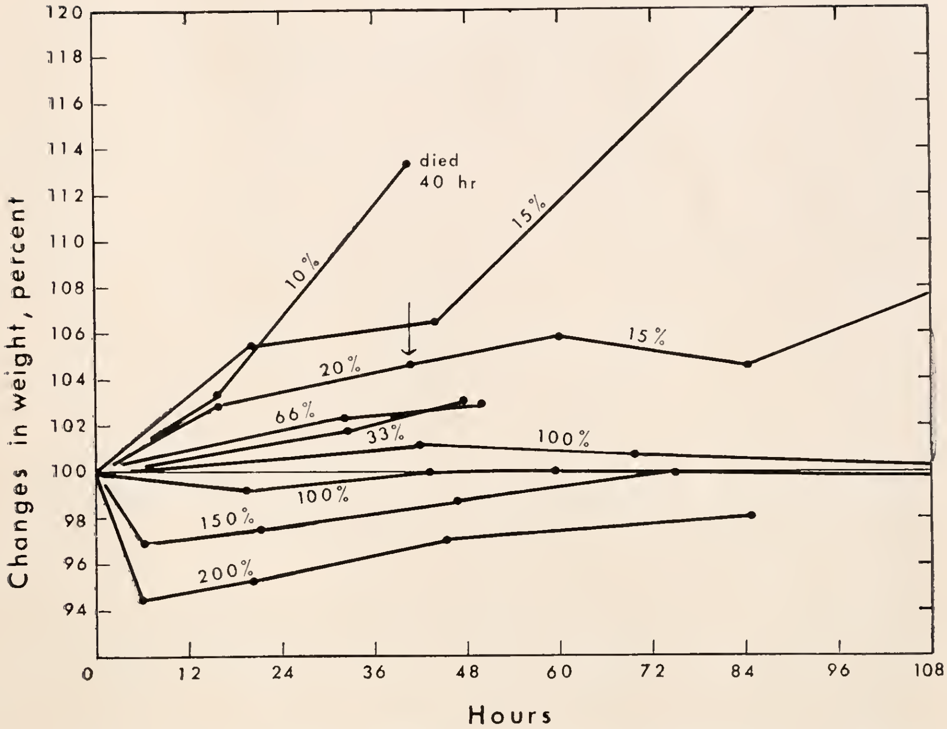


FIGURE 2. Changes in weight of *Limulus* of 92–118 g after direct transfer to different concentrations of sea water. Points are for individual specimens, except in 200% (mean of 3), 150%, 33% and 66% (mean of 2).

(Table II). There is also clear evidence for an increased hydration of the muscles at reduced salinity, animals in sea water of 33% and below having values in the eighties, compared with 79.1–79.7% in 50% and 66% sea water. A reduction in hydration from the normal range of 73.8–77.1% is found in specimens acclimatized

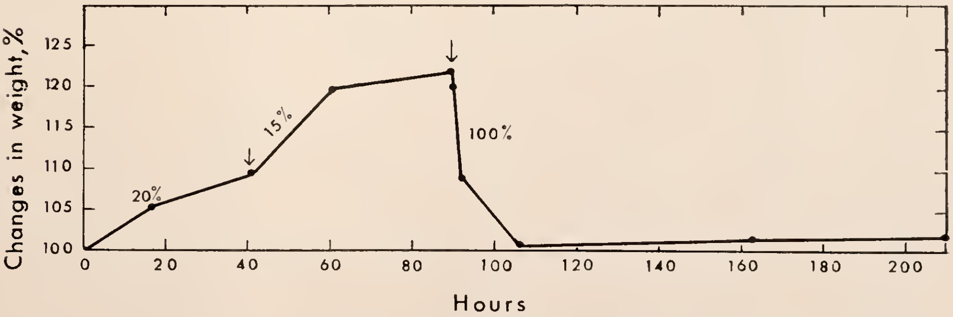


FIGURE 3. Changes in weight of a specimen of 103.8 g in various concentrations of sea water.

TABLE II

Water content of whole animals and of muscle in relation to salinity of external medium (5–200% sea water)

Medium %	Percentage water				Mean serum osmolality (milliosmoles)
	Whole animal	S.D.	Muscle	S.D.	
200	—	—	67.2 (3)	1.86	1931
150	—	—	73.2 (2)	1.71	1382
100	76.8 (3)	1.03	75.2 (4)	1.37	953
66	—	—	79.5 (2)	0.28	709
50	—	—	79.2 (3)	0.41	587
33	—	—	83.0 (2)	0.28	476
25	—	—	82.2 (4)	2.04	372
20	—	—	80.7 (1)	—	435
15	—	—	84.9 (2)	1.20	176
10	—	—	82.5 (2)	1.41	295
5	86.9 (2)	2.55	—	—	272

Weight of specimens mostly within range 102–139 g, but two in 5% were 21.1 and 32.3 g. Serum osmolality and muscle water contents are from the same specimens.

to 150% and 200% sea water, the ranges being 72.1–73.2% and 65.9–69.3%, respectively. The muscles are bathed in interstitial fluid and plasma, not sea water, and from the last column in Table II it is seen that serum osmolality and muscle hydration are inversely correlated (correlation coefficient—0.99). At the extremes of salinity, 100 g solids (chiefly protein and salts) are associated with 205 g water (muscle of 67.2% water) and 471 g (muscle of 82.5% water). In the medium of 15% where the two specimens increased to 116% and 122% of their initial weight, 100 g muscle solids is associated on the average with 562 g water.

TABLE III

Extracellular volume and extracellular water as measured by inulin space

Sex	Prosomal width (cm)	Weight (g)	Extracellular volume		Serum water (mg/ml)	Extracellular water (g/100 g)
			Actual (ml)	Per cent body weight		
♂	18.5	633.8	317.3	50.0	952	47.6
♂	9.5	106.7	52.1	48.8	948	46.3
♂	9.6	104.9	45.8	43.7	940	41.1
♂	9.4	100.3	64.3	64.1	947	60.7
♀	9.7	117.5	67.8	57.8	961	55.5
♀	9.5	101.0	53.8	53.3	950	50.6
♀	9.0	99.6	43.4	43.6	952	41.5
				Mean S.D.	Mean S.D.	Mean S.D.
				51.6 7.47	950 6.35	49.0 7.18

Time allowed for distribution of inulin after injection 5–10.3 hours. The difference in extracellular water between means for the males (48.9 ± S.E. 4.17) and females (49.2 ± 4.10) is not significant according to Student's *t*-test ($P = 0.7$). All specimens were from 100% sea water.

Whatever may be the mechanism for maintaining a more or less constant water content in muscle, it is clear that the general level of hydration is largely dependent on the osmotic concentration of the bathing fluid.

(3) *Extracellular water.* On the assumption that the inulin molecule does not penetrate cells, the data in Table III give an indication of the extracellular volume of specimens of 100–634 g, total extracellular water being then obtained by correcting for the solids in blood-serum. The mean value of 51.6 ml per 100 g for extracellular volume is high compared with a decapod crab such as *Carcinus maenas* in which a mean value of 32.6 ml has been found, using sucrose and inulin (Robertson, 1960b). Two features would partially explain this apparent marked difference: *Carcinus* has a relatively heavy calcified exoskeleton contributing to its weight, and also a lower water content, 65.4%, in the intermolt stage.

It is assumed that the extracellular water, 49.0 g average for a 100 g *Limulus,* includes water of the plasma, interstitial fluid round cells, and perhaps any fluid in the gut and in the coxal glands. On this assumption some 76.8–49 g, or 28 g, is the value for intracellular water and the water of the cuticle.

(4) *Permeability to water and salts.* All the foregoing data point to permeability to water of the body surface, probably mainly the lightly chitinized gills, water passing in when the animal is placed in a hypo-osmotic medium and out when placed in a hyperosmotic medium. But some slight drinking of the media has not been excluded physically by gut ligature. If it occurs it seems to be negligible as no large amount of fluid was seen in the gut during dissections.

Permeability to salts is not so self evident. However, from measurements of chloride in diluted sea water, particularly 5%, before and after specimens had been kept in it, it is clear that some chloride ions pass into the water before a steady state is attained. Permeability to salts can also be inferred from the changes in weight and measurements of osmotic concentration in animals in both stronger and dilute solutions. For example, if the 3 animals put into 200% sea water attained a new osmotic concentration, on average 1935 milliosmoles from an initial value of 955 (Table I), by water abstraction alone, the fall in weight would need to have been about 38% instead of the 6% values found (assuming a 77% water content initially—Table II). Salts must therefore have been absorbed to account for the rise in concentration of the blood to its new equilibrium value with the sea water.

Similarly, salts must have been lost to account for the decreased values of osmotic concentration in all the animals in diluted sea water. Taking the case of the 2 specimens in 33% sea water, a fall in blood concentration from 955 to 476 milliosmoles would require an osmotic intake of 38 g water per 100 g (the original weights were 114 and 117 g). Yet the average weight increase was 3%. Of all the specimens studied, one transferred directly to 15% sea water showed the greatest increase in weight, 94.6 to 115.0 g, 21.6% after 113 hr, and had an osmotic concentration of 181 milliosmoles, the second lowest recorded for any of the experimental animals. The theoretical value for a 100 g horseshoe crab of pure water which would have to be absorbed osmotically to reduce the concentration of blood and tissue water to 181 milliosmoles is (4.28 × 77) ml. It is quite obvious that the reduction cannot be due to osmotic uptake of water but must be effected chiefly by the outward diffusion of salts.

Of 29 *Limulus* tested in diluted sea water (66–5%), there were 7 deaths, all of medium-sized specimens (98–105 g) except one (about 35 g). Four of these, including the small one, died in 36–46 hr after direct transfer to 5% sea water, even though three were active and healthy for the first 25 hr. "Healthy" in this context means showing the normal swimming and aeration movements of the walking legs and opisthosomal flaps, well maintained tone of the flexor muscles, and, in the small one, ability to right itself in a few minutes. The final chloride content of 3 specimens in 5% sea water in different physiological condition after 40 hr, expressed as % of sea water chloride, was 636% (good condition), 331% (poor condition) and 222% (dead). Obvious increases in permeability to chloride have occurred in the last 2 individuals.

The other 3 deaths occurred on direct transfer from sea water to 10% (a 104.9 g animal which increased in weight 13.3% in 40 hr), on transfer to 10% after a preliminary 16 hr in 50% (100.8 g, 18.8% weight increase in 64 hr), and on direct transfer to 15% (98.0 g, 15.4% weight increase in 96 hr). In all 3 cases there was a gradual increase in water uptake until death, not a sudden increase at later stages.

Hardiness of a specimen of 97.2 g (♀) kept in running tap-water was shown by the fact that it increased by only 3.9% in 24 hr. This was partly due to the death and loss of its several epizoic triclad planarians (*Bdelloura*) and the loss of feces, as it had increased by 6.9% at the end of 4 hr. After 25 hr, the limbs still showed movements and the resistance to straightening of the flexed animal outside water had only slightly decreased from normal. Placed on its back in the tank it righted itself in 15 minutes. A small sample of blood showed a chloride value of 5.0 mg/ml, about that of animals living in 5–10% sea water. It was put back into 100% sea water and the subjective observation made that the resistance of the folded opisthosoma was back to normal in 25 minutes. Five hours later the weight had fallen to 99.5% of the original weight and the chloride had risen to 7.98 mg/ml. Inadvertently, the animal was left overnight on the bench after weighing and the final chloride equilibrium could not be followed.

Inorganic ions

(1) *Composition of serum and of flexor and extensor muscles of the prosoma-opisthosoma in animals from normal sea water.* Three comparisons of serum, muscle and the sea water in regard to inorganic ions, lactate and phosphate show fairly good agreement (Table IV). The sum of investigated ions in muscle comes to about half of those in serum and sea water. Apart from higher potassium and lower magnesium and sulphate concentrations in the serum compared with sea water, only small differences in the relative values of sodium and chloride are found, chloride being near the equilibrium value, sodium on average 1% higher, while calcium is higher in two cases, lower in the third.

In muscle half the osmotic concentration must be made up by organic compounds other than those of phosphorus. The increased potassium of muscle, about 9 times the serum value, is offset by lower concentrations of sodium and chloride (about a third to a quarter of serum values), magnesium (about a half), and calcium and sulphate (a half to a third). The average total acid-soluble phosphorus value of 73.3 mg atoms will presumably contain major fractions of arginine phosphate, ATP and hexose phosphates, compounds not separately estimated, acting as anions in the muscle cells. Lactate concentrations were especially high in specimen (2), although no excessive muscular activity had been noted, and the serum value was less than that of the third specimen.

Regarding cation-anion balance in the serum the mean value for cations is 569 milliequivalents, as against 543 for anions. The difference is presumably made up chiefly by protein anions and bicarbonate. For muscle the calculation is difficult, involving several undetermined factors such as the internal pH of the cells which influences the valency of the phosphate compounds, the proportions of the various phosphate compounds, how far the soluble proteins of the cells are acting as anions, and the possible presence of free arginine acting as a cation. Then there is the question of possible ion-binding to cell organelles. Simply assuming a valency of 1 for the total P atoms gives 259 milliequivalents anions as against 274 cations. A higher average valency would be more correct. In the case of the decapod crustacean *Nephrops*, average values calculated from knowledge of its phosphate compounds are 1.23–1.44 depending on the actual cell pH within the range of 6.0–7.0 (Robertson, 1961). Use of an average valency near the lower figure would give apparent cation-anion balance in *Limulus* muscle.

TABLE IV

Ionic composition of Limulus *blood-serum and whole muscle*

	mg-ions/kg water									Water (g/l or g/kg)
	Na	K	Ca	Mg	Cl	SO_4	Lactate	Total P	Total mg-ions	
♂ (1) Serum	439	10.9	8.8	46.3	507	14.8	0.16	0.15	1027	977
Muscle	147.4	89.0	4.25	22.4	190	—	9.8	58.1	521	771
♂ (2) Serum	441	13.3	10.6	47.5	516	11.6	0.18	0.74	1041	926
Muscle	91.8	106.1	3.20	17.8	142	3.9	35.1	89.6	490	749
♂ (3) Serum	454	11.3	10.2	44.3	519	16.0	0.59	0.40	1056	951
Muscle	139.4	103.4	3.83	21.4	146	—	12.4	72.3	497	770
Means										
Serum	445	11.8	9.9	46.0	514	14.1	0.31	0.43	1041	951
S.D.	8.2	1.3	0.30	1.6	6.2	2.3	0.24	0.30	14.5	25.5
Muscle	126.2	99.5	3.76	20.5	159	3.9	19.1	73.3	503	763
S.D.	30.1	9.2	0.53	2.4	26.6	—	14.1	15.8	16.3	12.4
Sea water	440	9.4	9.7	50.2	514	26.5	—	—	1050	990

(2) *Apparent extracellular spaces in muscle.* Chloride and inulin spaces were obtained by expressing the concentrations of these substances in muscle as percentages of those in serum, both on a water-content basis. The chloride space is a theoretical maximum for total weight or volume of muscle water which is extracellular, on the assumptions that serum has the same chloride content per unit of water as plasma and any interstitial fluid, and that chloride is absent from muscle cells. Any intracellular chloride would result in the chloride space as defined being larger than the extracellular space.

Results in Table V show that the inulin injected into the plasma distributes itself into 22.8% of the muscle water on the average, in 5–10 hours. This mean value is 75% of the chloride space, suggesting that there is some chloride inside the cells. Reasons for accepting the inulin space as a measure of extracellular space in muscle are the large size of the inulin molecule (MW *ca.* 5000), restricting its entry into cells, and its apparent suitability for measurement of extracellular space in such other invertebrates as decapod crustaceans and molluscs (Robertson, 1961; 1965).

The presence of haemocyanin in *Limulus* blood suggests that measurements of copper in muscle and serum will give a maximum value for the amount of blood plasma present in the muscle, but the possibility of other copper compounds in muscle, which would make this maximum an overestimate, must be accepted. Two estimates of copper spaces, expressed as percentage of total muscle water, were 3.5% and 15.4%, not in good agreement. The minimum value for interstitial fluid may be taken as about 13% (22.8–9.5%) of total extracellular water.

TABLE V

Apparent extracellular spaces in Limulus *muscle (concentrations in muscle as percentages of those in serum, on water content basis)*

Specimen no.	Extracellular spaces as per cent total muscle water		Muscle chloride (mg-ions/kg water)	Time allowed for distribution (hours)
	Chloride	Inulin		
1 ♂	27.8	24.9	145.9	5
2 ♂	28.3	19.4	147.5	7.5
3 ♂	28.9	19.4	152.6	8
4 ♂	31.7	25.5	167.3	10.3
5 ♀	30.2	22.3	153.5	6
6 ♀	36.2	25.6	192.1	6
7 ♀	28.8	22.2	152.9	10.3
Mean	30.3	22.8	158.8	
S.E.	1.10	1.01	6.13	

(3) *Intracellular ionic composition of muscle.* Calculations of intracellular concentrations are obtained by subtracting from the concentrations per kilogram muscle water the values for extracellular fluid. Of the two calculations in Table VI, the first one was made by subtracting the values for 249 g extracellular fluid (here taken as serum) from the concentrations per kilogram muscle water of the third specimen in Table IV, in which both extracellular fluid and chemical analyses had been obtained on the same specimen. The remaining concentrations in 751 g muscle water were then expressed as cell values per kilogram water. For the second calculation the means of the other two animals in Table IV were used, in conjunction with the mean extracellular space, 22.8%, of Table V.

The two sets of values for ions in the muscle cells do not differ greatly, those for sodium and chloride the most. Because of the subtraction of sodium- and chloride-rich extracellular fluid, the concentrations of these ions fall to about a quarter of those in whole muscle. Magnesium still remains at a higher intracellular concentration than calcium, although both are reduced. At the same time potassium and phosphate increase by about an eighth. The intracellular potassium concentration now exceeds the sodium, and is approached in concentration only by phosphate; both these features are found in the few invertebrate muscles analyzed in the same way. The final result is a reduced total concentration of ions, about 32% of the serum concentration, compared with 48% in whole muscle.

Comparison of cell/serum ratios in Table VI shows that potassium, lactate and phosphate are accumulated in the cells, the latter especially, as it is the principal anion balancing most of the potassium. Chloride and the three other cations are reduced, magnesium the least.

The mean chloride ratio of 0.084 is not very different from that which would exist if there is a Donnan equilibrium between the concentrations of potassium and chloride inside and outside the cells: $\mathrm{K}_i/K_o = 11.0$, $\mathrm{Cl}_o/\mathrm{Cl}_i = 11.9$. But the mean chloride value inside the cells is derived from two values each of which gives ratios considerably above and below this, so that a simple Donnan relationship is improbable.

TABLE VI

Intracellular composition of muscle compared with blood-serum

	mg-ions/kg water							
	Na	K	Ca	Mg	Cl	Lactate	Total P	Total
Muscle cells								
(1)	35.2	134	1.72	13.8	22.4	16.3	96.2	320
(2)	22.4	123	1.97	12.2	63.9	29.0	95.5	348
Mean	28.8	129	1.85	13.0	43.2	22.7	95.7	334
Serum								
(1)	454	11.3	10.2	44.3	519	0.59	0.40	1040
(2)	440	12.1	9.7	46.9	512	0.17	0.45	1021
Mean	447	11.7	10.0	45.6	516	0.38	0.43	1031
Ratio: $\frac{\text{cell}}{\text{serum}}$	0.064	11.0	0.19	0.29	0.084	59.7	223	0.32

First calculation based on ♂ specimen in which both inulin space and muscle composition was measured; second calculation using mean inulin space (22.8 per cent of muscle water) and mean data for other two ♂ specimens.

Average water content: cells 727 g/kg, serum 951 g/liter.

(4) *Composition of serum before and after dialysis against sea water.* Since *Limulus* blood contains the protein haemocyanin and the gills are apparently permeable to the inorganic ions of sea water, it is possible that a Donnan equilibrium exists with respect to at least certain ions, those cations in higher concentration in the plasma than in sea water, and those anions in lesser concentration in the plasma.

A sample of serum in Visking viscose cellulose tubing was dialyzed against sea water of almost exactly the same concentration as that from which the animal was taken. After dialysis for 24 hr, the serum was analyzed for comparison with a sample of the original serum. The final pH of the sea water was 8.0.

For the cations and anions, excluding calcium, of the dialyzed serum, the mean Donnan ratio is 1.018 (Table VII). The much higher value of calcium in the serum, 115.5%, of that in the sea water, indicates the presence of some calcium bound in an unionized complex with protein. This is a usual feature in blood containing significant quantities of protein. A small proportion of magnesum may be bound, about 4% of the total, if the mean Donnan ratio is applied to the figure of 108.0%, the concentration of magnesium in the serum after dialysis. A slight anomaly is apparent, in that the chloride of the dialyzed serum expressed in relation to water content was never less than the chloride of the sea water expressed similarly, whereas it should have been nearly 2% less according to the Donnan relationship. This occurred despite careful replicate analyses.

Taking comparison of original serum with dialyzed serum as a measure of ionic regulation, it is clear that in the blood of the specimen analyzed sodium and potassium are regulated at values higher than those of physico-chemical equilibrium (sodium only slightly), that calcium, magnesium and sulphate are regulated at levels lower than those of equilibrium, leaving chloride as the only ion in diffusion equilibrium.

TABLE VII

Composition of serum before and after dialysis against sea water

	mg-ions/kg water						g/liter
	Na	K	Ca	Mg	Cl	SO_4	Water
Original serum	454	11.3	10.2	44.3	519	16.0	951
Serum after dialysis	450	9.5	11.2	54.3	517	25.4	950
Sea water	441	9.4	9.7	50.3	515	26.5	990
Serum as per cent dialyzed serum	100.9	118.9	91.1	81.6	100.4	63.0	—
Dialyzed serum as per cent sea water	102.0	101.1	115.5	108.0	100.4	95.8	—
Donnan ratio*	1.02	1.01	—	1.04	1.00	1.02	—

* $\frac{Na_s}{Na_{sw}} = \frac{\sqrt{Mg_s}}{\sqrt{Mg_{sw}}} = \frac{Cl_{sw}}{Cl_s}$ *etc.* Protein = 40.0 g/liter. Mean ratio = 1.018.

By considering also the other two detailed analyses of *Limulus* serum in Table IV, a general conclusion would be that *Limulus* regulates the ionic composition of its blood by reducing its calcium, magnesium and sulphate on the average some 12%, 12% and 43%, respectively, below the values obtaining in a physico-chemical equilibrium with sea water, while maintaining sodium and chloride within about 1–2% of equilibrium. Potassium shows the most variable regulation at about 24% above dialysis equilibrium (range 11–39%). Possible participation of the coxal glands and the gills in this ionic regulation has not been investigated.

(5) *Effect of salinity on composition of serum and muscle.* When *Limulus* is allowed to come into equilibrium with sea water of greater strength than normal, the blood becomes isosmotic (Table I). In Table VIII it is seen that in 150% sea water the pattern of ions in the serum is very similar to that in normal sea water, only potassium and calcium showing more than slight changes, in both cases a relative fall in level, more pronounced in the case of the latter. However, when in 200% sea water, the differences in ions between internal and external media practically disappear, essentially remaining only in sulphate concentration which in the serum is 85% of that in sea water, compared to 53% and 55% in 100% and 150% sea water.

Table VIII

Changes in composition of serum in relation to salinity of external medium

Sea water (per cent)	Medium	mg-ions/kg water						
		Na	K	Ca	Mg	Cl	SO_4	Total ions
200	Serum	921	19.7	23.3	103.5	1079	45.9	2192
	Sea water	924	19.7	22.9	102.3	1087	54.3	2210
	Serum as per cent sea water	100	100	102	101	99	85	99
150	Serum	627	15.0	13.2	62.7	721	20.5	1459
	Sea water	624	13.8	15.4	71.5	736	37.4	1498
	Serum as per cent sea water	100	109	86	88	98	55	97
100	Serum	445	11.8	9.9	46.0	514	14.1	1041
	Sea water	440	9.4	9.7	50.2	514	26.5	1050
	Serum as per cent sea water	101	126	102	92	100	53	99
50	Serum	297	5.69	6.86	28.1	326	6.7	670
	Sea water	225	4.78	4.93	25.7	263	13.5	537
	Serum as per cent sea water	132	119	139	109	124	50	125
20	Serum	224	7.88	5.66	13.7	222	2.5	476
	Sea water	87.4	1.86	1.92	10.0	102	5.3	209
	Serum as per cent sea water	256	424	295	137	218	47	228
10	Serum	156	3.80	3.28	6.5	153	2.0	325
	Sea water	45.8	0.98	1.00	5.2	53.5	2.8	109
	Serum as per cent sea water	341	390	328	125	286	71	298

Figures are means of analyses of two animals in 200 per cent sea water, of three in 100 per cent, and of single specimens in the other concentrations.

In the three diluted sea waters, the serum remains hyperosmotic, the percentage difference in total ions between it and the sea water increasing with dilution from 25% to 198%. Individual ions in the serum do not vary in accordance with the total concentration, each being apparently controlled separately. Sodium and chloride form 93–95% of the total ions in the serum (91–92% in 100% sea water and greater strengths). With dilution sodium rises slightly as a proportion of the total, chloride remaining nearly proportional. In the lower salinities serum potassium and calcium increase relatively more than sodium (although these ions fall in absolute concentrations), while magnesium and sulphate fall considerably in both absolute and relative terms.

Muscle concentrations of inorganic ions rise or fall with the concentration of the blood and the surrounding medium (Table IX). It is easy to visualize, say, an increase in salinity causing increases in blood ions which are then reflected by a final higher concentration of muscle ions; and similarly for decreases. But the concentrations of phosphate are more difficult to relate to the osmotic concentration of the blood. Being for the most part not easily diffusible compounds (except inorganic P), phosphates would be expected to be more stable in amount at different salinities, and certainly should not change on account of the negligible effect of minute quantities of phosphate in sea water. From the data for phosphorus in Table IX, it would appear that there is little fall in concentration in 20% and 10% sea waters; as salts are lost in the low salinities the phosphate rises as a percentage of total ions from 15% in full-strength sea water to 31% of the total in 5%. Although the absolute amount of phosphate, 132 mg-atoms, in one of the 200% specimens shows an 80% rise from the average of 73.2 in normal animals, it falls as percentage of the total ions to 10%.

It is this increase of phosphate to 132 and 137 mg-atoms that is puzzling, since it is far more than would be expected from the decrease in water content of the muscle, unless the specimens had, in normal sea water, higher muscle values than the highest (89.6 mg-atoms) of the 3 specimens with 73.3 mg-atoms as mean. While this is not impossible, phosphate may perhaps have been transferred to the muscle cells from other tissues, such as the hepatopancreas.

Table IX

The composition of muscle in relation to serum as influenced by salinity of external medium

Sea water (per cent)	Tissue	mg-ions/kg water							g/kg or g/l
		Na	K	Ca	Mg	Cl	P*	Total ions	Water
200	Muscle (1)	362	181	15.4	53.9	568	132	1312	663
	(2)	370	183	17.3	50.3	—	137	(757)	659
	Serum (1)	921	18.7	21.4	102.7	1064	0.31	2128	929
	(2)	921	20.7	25.2	104.3	1094	0.47	2166	945
	Ratio: muscle/serum (1)	0.393	9.68	0.720	0.525	0.534	426	0.617	—
	(2)	0.402	8.84	0.687	0.482	—	291	—	—
	Mean	0.398	9.26	0.704	0.504	0.534	359	0.617	—
100	Muscle Mean†	126	99.5	3.76	20.5	159	73.3	482	763
	Serum Mean†	445	11.8	9.9	46.0	514	0.43	1027	951
	Ratio: muscle/serum	0.283	8.43	0.380	0.446	0.309	170	0.469	—
20	Muscle	46.1	89.5	1.88	12.6	47.4	76.0	273	807
	Serum	224	7.88	5.66	13.7	222	0.43	474	908
	Ratio: muscle/serum	0.206	11.35	0.332	0.92	0.214	177	0.576	—
10	Muscle	32.8	59.3	1.66	9.5	36.5	63.1	203	815
	Serum	156	3.80	3.28	6.5	153	0.35	323	974
	Ratio: muscle/serum	0.210	15.6	0.51	1.46	0.239	180	0.628	—

* Mg-atoms trichloracetic acid—soluble P.
† From Table IV.

Muscle/serum ratios in the altered sea waters show the same general features as the ratio in normal sea water, high values for potassium and phosphate, low values for the remaining ions. As salinity and serum concentrations decline, so do the muscle/serum ratios of sodium and chloride, but from a base-line of 100% sea water the ratios of potassium, magnesium and phosphate increase with dilution, that of phosphate only slightly. The end result is a slight increase from 0.47 to 0.63 in the ratio of muscle/serum total ions.

It is obvious that the ratios between the ions of muscle and serum are kept more constant over the range of salinity tested than the ions themselves. The highest ratios are never more than two or three times those of the lowest, but individual values for ions vary widely. For example, comparing muscle from *Limulus* in

10% and 200% sea waters, the highest values of sodium, calcium and chloride are, respectively, eleven, ten and sixteen times those of the lowest. This greater constancy in the ratios is due to the fact that the ions of the serum are varying in the same direction, influenced by the external salinity, and in fact are broadly responsible for the changes in muscle ions.

Nitrogenous constituents of muscle

In Table X are shown analyses of nitrogenous compounds and ammonium ions in the muscles of some of the same animals for which data on inorganic ions and total phosphate have already been given (Tables IV and IX). Total non-protein nitrogen of muscle, expressed as mg-atoms, does not differ much in the animals from 200% and 100% sea water, but is smaller in amount in the two animals in dilute sea water. The figures for the 3 specimens in normal sea waters, 538–757 mg-atoms, agree well with those of Bricteux-Grégoire *et al.* (1966), 462–776, although the latter are for hydrolyzed dialysates of homogenized muscle, whereas the figures in Table X are for tungstic acid extracts of muscle. In dilute sea waters the values are 406 and 235 (Table X) which may be compared with one of 271 mg-atoms given by the Belgian authors for an animal adapted to sea water of Δ 0.79° C (about 44% of concentration of Woods Hole sea water).

TABLE X

Nitrogenous constituents of muscle

Sea water (per cent)	mM/kg water						
	Free amino acids			Trimethylamine oxide	Urea	Ammonium	Total non-protein N†
	Total α-amino N	Arginine*	Proline				
200	316.7	—	55.7	—	—	—	743
100 (1)	82.3	27.2	2.7	3.9	0.8	6.7	538
(2)	128.1	61.2	25.1	4.9	0.4	2.9	756
(3)	103.4	41.5	6.9	5.2	—	5.6	757
Mean	104.6	43.3	11.6	4.7	0.6	5.1	684
20	88.5	49.4	5.4	4.4	0.5	4.1	406
10	67.6	25.5	0.6	1.7	1.1	3.1	235

* Probably mostly as arginine phosphate in the living animal.
† Mg-atoms.

A surprising feature is the relatively low concentration of free amino acids, an average of 105 m*M* in *Limulus*, compared with much higher values in decapod crustaceans (such as 512 m*M* in *Nephrops* (Robertson, 1961)). Included with the amino acids is taurine whose α-amino N reacts with Folin's amino acid reagent. The ratio of amino acids to total nitrogen increases in the muscles of animals in 200% sea water and in 10%. After taurine in concentration come arginine and proline, the latter rather variable, the former more consistent with values between 26 and 61 m*M*. Compared with *Nephrops*, *Limulus* has a small amount of proline in its muscles, and about half the amount of arginine.

Urea is present only in trace quantities and trimethylamine oxide to the extent of 5 m*M*. Ammonium ions are also present, but in small quantity. Some may have arisen by release of ammonium from compounds such as amides after the muscle extracts had been made.

Allowing for the 4 N atoms of arginine, and adding to the amino acid- and taurine-N the other estimated N- compounds plus ammonium, the sum still comes far below the total non-protein N, leaving 64% of it undetermined in the animals in full-strength sea water, 39% and 36% undetermined in the 20% and 10% diluted waters. Even taking into account the 5 N atoms of the adenosine phosphates which were not estimated, there would still remain more than half of the total N atoms still unallocated to known compounds in the muscles of animals in normal sea water, and about a quarter in the case of the specimen in 20% sea water.

Bricteux-Grégoire *et al.* (1966) who studied some *Limulus* in full-strength sea water (Δ 2.09° C) and in diluted sea water (Δ 0.78° C) have given details of individual amino acids in blood serum (total 0.7–1.5 m*M*/liter) and muscle. They also point to the small contribution of amino acids and taurine to the total dialyzable N, leaving unknown nitrogenous compounds playing a significant role in maintaining the osmotic concentration of the cells.

Osmolality

From the data given in the two previous major sections, the osmolality of serum and muscle can now be calculated at 4 salinities and that of muscle cells at normal salinity. Neglecting for the present the osmotic coefficients of the salts and nitrogenous compounds, and the possibility of some ion binding to proteins, a summation of inorganic and organic ions and of nitrogenous constituents should give osmolality, as the concentrations are per kg solvent water (Table XI).

TABLE XI

Osmotic concentration of serum, whole muscle and muscle cells

Constituent	mg-ions or mM/kg water								
	200 per cent sea water		100 per cent sea water			20 per cent sea water		10 per cent sea water	
	Serum	Muscle	Serum	Muscle	Muscle cells	Serum	Muscle	Serum	Muscle
Sodium	921	366	445	126.2	28.8	224	46.1	156	32.8
Potassium	19.7	182	11.8	99.5	129	7.9	89.5	3.8	59.3
Calcium	23.3	16.4	9.9	3.76	1.85	5.7	1.88	3.3	1.66
Magnesium	103.5	52.1	46.0	20.5	13.0	13.7	12.6	6.5	9.5
Ammonium	—	—	—	5.1	6.6	—	4.1	—	3.1
Chloride	1079	568	514	159	43.2	222	47.4	153	36.5
Sulphate	45.9	—	14.1	3.9	0.9	2.5	—	2.0	—
Lactate	—	—	0.31	19.1	22.7	—	21.3	—	6.5
Phosphate	0.39	135	0.43	73.3	95.7	0.43	76.0	0.35	63.1
Total ions	2193	1320	1041.5	510.4	341.8	476.2	298.9	325.0	212.5
Amino acids	—	317	—	105	136	—	89	—	68
Trimethylamine oxide	—	—	—	4.7	6.1	—	4.4	—	2.1
Urea	—	—	—	0.6	0.8	—	0.5	—	1.1
Remaining non-protein N	—	426	—	439	568	—	160	—	85
Total	2193	2063	1042	1060	1053	476	553	325	369

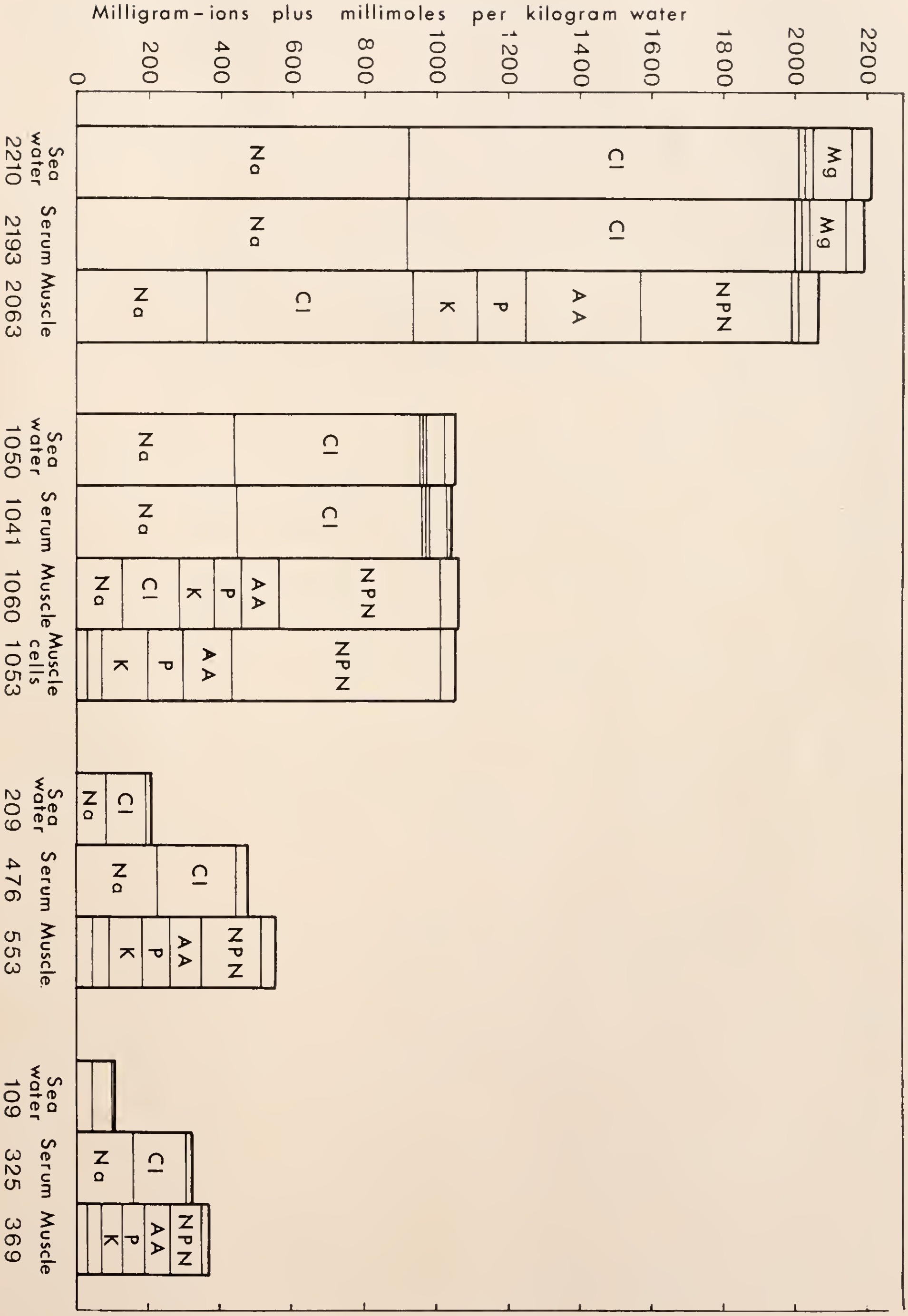


FIGURE 4. Composition of *Limulus* serum and muscle in relation to concentration of the external medium; AA—amino acids; NPN—remaining non-protein nitrogen.

The agreement is good (within 2%) between serum, muscle and muscle cell concentrations in the animals from 100% sea water, less good in the comparison of muscle and serum at the other salinities, in which the muscle concentrations exceed the serum by 14–16% in the specimens from dilute sea water, and are 6% below in the specimens from 200% sea water.

Figure 4 shows the changes in the principle constituents at the 4 concentrations studied.

Apart from the uncertainties of ion binding, several inaccuracies leading to overestimates exist in the muscle data: the phosphate is really in mg-atoms as the fraction of ATP (with its 3 P atoms) was not determined; the remaining non-protein N includes the 5 N atoms of ATP; any arginine phosphate is included both under amino acids and phosphate; the remaining non-protein N compounds may (like the adenosine polyphosphates) contain more than 1 atom N per molecule. So more complete knowledge would tend to reduce the total osmotic concentration of muscle as estimated.

A more accurate estimate of osmolality would be obtained if osmotic coefficients for sea water and for organic compounds are used (Robertson, 1961, 1965). Unfortunately, lack of published data on the osmotic coefficients of most of the amino acids makes this impossible even when the concentrations of each amino acid in the muscle are known, and even the identity of the other nitrogenous compounds in muscle has not been established. Perhaps it is best at present to assume an average coefficient of 1 for all the nitrogenous compounds.

For inorganic salts the osmotic coefficients are well below that for ideal solutions, and for sea water osmotic coefficients can be calculated from the osmotic equivalence data of Robinson (1954) and the g-ionic concentration of sea water (1.1368 at a chlorinity of 19.00‰) as given by Sverdrup, Johnson and Fleming (1942). For example, the osmotic coefficient for a sea water of Cl 19.00‰ is 1027.3/1136.8, the first figure being the concentration in milliosmoles and the second figure milligram-ions by analysis. This value is 0.904, and corresponding values for sea waters of Cl 22.00‰ and 10‰ are 0.908 and 0.897. For 200% sea water the osmotic coefficient may be calculated to be 0.92.

It is reasonable to assume that the predominantly inorganic ions of *Limulus* serum have proportionally the same osmolar concentration as the ions of sea water and that the appropriate osmotic coefficients can be applied. It is further assumed that the inorganic ions and phosphates of the muscle would behave approximately like serum ions. In the calculations of the milliosmolar concentrations of Table XII no allowance has been made for possible ion binding. This is negligible in the serum, but probably significant in the muscle; its extent in muscle remains a subject for future research. The final calculations show that in all cases except the 200% sea water specimens (for which only one N-analysis is available) the muscle has an apparently higher osmotic concentration than the serum, about 7–8% higher in both muscle cells and whole muscle, some 18–22% higher in the specimens from 10% and 20% sea water. Possible reasons for this have already been discussed. There is no reason to doubt that the blood and muscle are isosmotic. Direct measurements of osmolality of the sera by the Krogh-Baldes thermoelectric method show good agreement with the calculated totals. The slightly lower totals would have been increased by a few milliosmoles if estimations had been made of amino acids (*ca.* 0.7–1.6 m*M*, Bricteux-Grégoire *et al.*, 1966) glucose (*ca.* 0.2 m*M*, Tamar, 1965) and bicarbonate (7 mg-ions, Homer W. Smith in Cole, 1940).

DISCUSSION

When comparisons are carefully made of the blood of *Limulus* which have been equilibrated with normal Woods Hole sea water, 31–32‰, the serum has been found to be isosmotic within 1% (freezing-point data of Garrey, 1905 and of Dailey, Fremont-Smith and Carroll, 1931, also the present data using a thermo-electric vapor pressure method). Cole (1940) states that 6 pooled samples of *Limulus* blood collected over 3 summers were "consistently hypertonic" and thinks that this is the normal condition. The quoted values for animals kept 3 months in Salsbury Cove (Maine) sea water are serum Δ 1.783° C, sea water 1.759° C, a difference of 1.4%. He also gives data for specimens from the New York Aquarium analyzed by Smith, serum Δ 1.880° C, sea water 1.850° C, a difference of 1.6%. It is difficult to judge the weight to be given to these values since, if they are means, no tests of significance are given or estimates of errors of measurement. Although no differences between the sexes were found in the osmotic concentration of the blood from animals in 100% sea water (Table I), there is a significant difference at the level of $P = 0.05$ between the means of the 10 values for sea water, 951 ± 0.298 and those for serum, 955 ± 1.303. Thus the blood is slightly hyperosmotic, by 0.4%, and it may have been approximately so in the specimens studied by Cole and Smith. A conclusion that the blood of *Limulus* is isosmotic with sea water of salinity 31–34‰ within 1.5% would practically cover the data.

TABLE XII

Osmolality of serum, whole muscle and muscle cells

	milliosmoles/kg water								
	200 per cent sea water		100 per cent sea water			20 per cent sea water		10 per cent sea water	
	Serum	Muscle	Serum	Muscle	Muscle cells	Serum	Muscle	Serum	Muscle
Inorganic ions and phosphate *etc.*	2018	1214	940	461	309	429	269	293	191
Amino acids	—	317	—	105	136	—	89	—	68
Trimethylamine oxide and urea	—	—	—	5	7	—	5	—	3
Remaining non-protein N	—	426	—	439	568	—	160	—	85
Total	2018	1957	940	1010	1020	429	523	293	347
Direct measurement	2017	—	953	—	—	435	—	299	—

McManus (1969) using a comparative melting-point method found that horse-shoe crabs from Delaware Bay were approximately isosmotic when kept in tanks of running bay water of about 25‰ salinity. At this salinity the osmotic equivalence should be 0.40 *M*-NaCl and from one of his figures the bay water seems to be only 0.34 *M*. He claims considerable fluctuations among his 8 large male specimens in this water, both among individuals and at different times of successive sampling (8, 16 and 24 hr). Two points on the figure suggest that the blood may become hypo-osmotic (0.31–0.32 *M*) after 8, 16 and 24 hr in the water. This seems very improbable, and shows, perhaps, a limitation in accuracy of the melting-point technique. Dailey *et al.* (1931) stress the importance of comparing the blood with sea water each time equilibrium studies are made, as they found that *Limulus* blood reflected the dilution of sea water with rain water at Woods Hole, remaining isosmotic.

McManus (1969) found that large male crabs could withstand direct transfer to diluted water of about 13‰ salinity without any ill effects, but transfer to 6–7‰ and to deionized water caused swelling of the limb joints and gills, with bursting of the gill membranes in 4 out of 8 of the former and in the 4 latter. Osmotic concentrations measured up to 24 hr showed a rapid fall in 13‰ and 6–7‰ salinity but remained higher than the medium. Four specimens (118–479 g) transferred gradually from 25‰ to 3–4‰ salinity, with stays of 4 days in 13‰, and 2 days in 6–7‰ changed little in weight over this period, and became noticeably sluggish in the final dilution, although maintaining normal weight within 2% for 74 hr.

A salinity tolerance study on groups of 10 small *Limulus* (prosomal width 7.5 cm or less) over a period of 127 hr showed no deaths in diluted sea water of 12.5‰, but a rising mortality of 5%, 15%, 30%, and 40% in waters of 10.0‰, 7.5‰, 5.0‰ and 2.5‰, respectively, while the 20 kept in deionized water all died in 12–72 hr.

Apart from McManus's study the only published data on osmotic regulation in *Limulus* are isolated values such as those of Cole (1940) on a specimen from Delaware Bay, Δ 1.378° C, sea water 1.336° C; and on one kept in New York Harbor sea water, serum Δ 1.01° C, water 0.651° C (analyzed by Homer W. Smith). Bricteux-Grégoire *et al.* (1966) reported the mean value of Δ 0.85° C for medium sized specimens gradually acclimatized to diluted sea water of Δ 0.79°. At this sea water strength they survived at least 15 days. Garrey (1905) gave values of Δ 1.12° C in a specimen after 52 hr in sea water of Δ 1.02° C, and also found that the blood of a specimen kept for 16 hr in sea water concentrated by evaporation to give Δ 3.80° C was isosmotic at Δ 3.79° C.

The foregoing data are widely expanded and reinforced by the osmotic concentration figures given in Table I, from animals in 5–200% sea water, 47–2084 milliosmoles (about Δ 0.09°–Δ 3.87° C). *Limulus* is extremely tolerant of changes in salinity. Over a sea water range of 100–200% the blood concentrations are 954–2058 milliosmoles, the blood being isosmotic within 1.5%, while over the 5–66% range the blood is hyperosmotic, ranging between 176 and 718 milliosmoles in water of 47–630 milliosmoles.

If the osmotic concentration curve (Figure 1) is compared with that for the euryhaline decapod crustacean *Carcinus maenas*, common in European coastal waters and the Woods Hole region, the osmotic tolerance as determined experimentally is seen to be much the same, Δ 0.17° C–Δ 2.88° C (91–1550 milliosmoles) (Robertson, 1960b), but at the lowest salinity studied by Duval (1925), Δ 0.17° C, only 2 of 6 crabs survived for 20 hr, and those in water of Δ 0.38° C died in 24–48 hr. These were all direct transfer experiments with no intermediate acclimatization. In water of Δ 0.60° C (323 milliosmoles) and above the crabs seemed to survive indefinitely.

More recent studies of *Carcinus* from the North Sea (32‰) and the Baltic (13–18‰) have shown that in water of about Δ 0.33° C (178 milliosmoles) animals from the Baltic had the greater freezing-point depressions after 3 days, equivalent to about 538–754 compared with 452–592 milliosmoles. Cross adaptation to different habitat salinities for 2–3 weeks reduced but did not abolish these differences (Theede, 1969).

The main difference in osmoregulatory ability between *Carcinus* and *Limulus* is that the former maintains its blood at relatively higher osmotic concentration in low salinities. Theede's data are for a temperature of 16.5° C, Duval's (unspecified) probably at temperatures near those of *Limulus* in this paper, 21–23° C.

The San Francisco Bay population of *Rhithropanopeus harrisi*, a xanthid crab, is well adapted to brackish water situations. The melting-point value of the blood at a salinity of about 21 m*M*-NaCl, 39 milliosmoles, is near 232 m*M*, about 424 milliosmoles (Smith, 1967), a concentration considerably higher than the 220–335 maintained by small *Limulus* in a medium of 47–48 milliosmoles.

As mentioned in the introduction, *Limulus* has sometimes been found in waters of salinity 6–8‰ (18–24% of Woods Hole concentration). Whether these are young or adult animals, or whether the stay in such diluted water is a seasonal phenomenon in the particular areas, it seems from the data in Table I that even lower salinities could be tolerated for some time, especially if the animals faced a gradual acclimatization.

Present and former analyses of blood-serum of *Limulus* are compared in Table XIII. Considering the varied methods by which the results were obtained and the possibility of systematic and other errors, the results are not too different. Macallum's (1910) analysis mostly in triplicate, using classical gravimetric techniques, is perhaps closest to those in this paper. Cole (1940) admits a systematic error in his magnesium method leading to low results (−30% in the analysis of sea water) and he attributes values of 0.0629 and 0.0609 given by Smith for the SO_4/Cl ratio in Salsbury Cove and New York sea water (+22% and 18% on the standard accepted value) as perhaps due to normal variation in near-shore sea water. This is improbable. Cole's Na/Cl ratio for sea water, 0.931, is too high by 9% and so possibly are his ratios for *Limulus* serum. For the methods used by the author a series of estimations on synthetic sea waters gave standard deviations from the theoretical for Na, K, Ca, Mg, Cl and SO_4 of 0.28%, 1.04%, 0.81%, 1.36%, 0.06% and 0.60%, respectively.

In diluted sea waters the general finding is a decrease in the Mg/Cl and SO_4/Cl ratios and an increase in that of Na/Cl. The K/Cl and Ca/Cl ratios of the serum remain larger than the respective sea water ratios.

If ionic regulation is defined as the maintenance of concentrations of ions differing from those of a passive equilibrium with the external medium, the pattern in *Limulus* (Table VII) is closest to that of the spider crab *Maia squinado* of the 14 decapod crustaceans examined in the same way (Robertson 1949, 1953). Only in its low calcium level, lower than that of dialysis equilibrium, does it differ from *Maia* and the other isosmotic decapods. This low level of calcium seems to be associated with lack of a calcified skeleton in *Limulus*.

In muscle of *Limulus* from normal sea water, inorganic ions and organic phosphates and lactate account for about 48% of the osmotic concentration, the remaining proportion being made up by non-protein nitrogenous compounds. This figure is practically identical with that in *Nephrops* (Robertson, 1961), but after subtraction of the extracellular fluid the proportion of ions in the muscle cells falls to 32% in *Limulus* compared to 40% in *Nephrops*. In animals from reduced salinities, the proportion increases to 54% and 58% in 20% and 10% sea waters, and also to 64% in animals from 200% sea water.

Table XIII

Composition of Limulus *blood-serum, according to various authors, milligram-ion* (*m*M) *ratios in relation to chloride*

Source of material and author	Cl (mg-ions/l)	Na/Cl	K/Cl	Ca/Cl	Mg/Cl	SO_4/Cl
Woods Hole Macallum, 1910	487	0.824	0.0272	0.0192	0.0873	0.0315
Woods Hole Dailey *et al.*, 1931	500	0.883	—	—	—	—
Maine Cole, 1940	478	0.956	0.0207	0.0195	0.0605	0.0442
New York Smith in Cole, 1940	463	0.909	0.0311	0.0346	0.0584	0.0445
Woods Hole	489	0.866	0.0230	0.0193	0.0895	0.0274
J. D. R.	310	0.958	0.0184	0.0221	0.0906	0.0216
Woods Hole	202	1.009	0.0355	0.0255	0.0617	0.0113
J. D. R.	149	1.020	0.0248	0.0214	0.0425	0.0131
Delaware Bay Cole, 1940	308	0.984	0.0282	0.0205	0.0799	0.0183
New York Harbor Smith in Cole, 1940	265	0.981	0.0249	0.0208	0.0194	0.0169
Sea water Sverdrup *et al.*, 1942	549	0.856	0.0181	0.0187	0.0976	0.0515

Decrease in salinity leads to a reduction in the amounts of non-protein nitrogenous constituents in *Limulus* muscle. This is a feature found also in euryhaline crustaceans (*e.g.*, *Carcinus*, Shaw 1958; *Eriocheir*, Bricteux-Grégoire *et al.* 1962) in which free amino acids may account for a third or a half of the osmotically active constituents in muscle. This proportion in *Limulus* is much less, as pointed out already by Bricteux-Grégoire *et al.* (1966), 6–9% and 10% (including taurine) in the muscles of their specimens in full strength and 40% sea water, and 12–23% in the present series over 200–10% sea water. This leaves a large fraction of the non-protein nitrogen still undetermined and unknown, as it must be attributed to compounds other than trimethylamine oxide and urea.

Finally, the wide tolerance of *Limulus* to increases and decreases of salinity must be of value to animals temporarily cut off in evaporating lagoons or shallows in the southern part of its range or in areas affected by freshwater outflows. Information is required on the salinity tolerance of younger stages of *Limulus* than those studied here or by McManus (1969), and on whether the appearance of specimens in brackish water is related to such factors as season, temperature and state of maturity which are likely to influence powers of osmotic regulation.

This work was supported by a grant GB-3447 from the National Science Foundation to the Department of Invertebrate Zoology, Marine Biological Laboratory. I wish to thank the then Director Dr. Philip B. Armstrong and Dr. W. D. Russell-Hunter for facilities during that part of the investigation undertaken at Woods Hole, and for kindness during my stay. I am indebted to Dr. Carl N. Shuster, Jr. for the benefit of his wide knowledge of *Limulus,* freely given. Dr. John H. Lochhead kindly read the proofs.

SUMMARY

1. Osmotic and ionic regulation of *Limulus* has been studied over a wide range of salinities. The blood is isosmotic in sea water of 100–200% (950–2084 milliosmoles) within 1.5%. It is hyperosmotic in dilute sea water of 10–66%. After a period of acclimatization in waters of 50–10%, some small horseshoe crabs (prosomal width about 7 cm) remained healthy in 5% sea water (48 milliosmoles, about 1.7‰ salinity) for 2–5 days, the blood in 4 specimens being kept at a level of 229–335 milliosmoles.

2. Analyses of blood-serum as originally obtained and after dialysis against sea water give the following measure of ionic regulation: Na 101%, K 119%, Ca 91%, Mg 82%, Cl 100% and SO_4 63%.

3. Weight changes in waters of different salinity have been studied and the final water content of the muscles. The latter ranges from 67% in specimens from 200% sea water to 83% in specimens from 10%.

4. From experiments with injected inulin solutions, the average extracellular volume in crabs of 100–634 g weight is 52% of the body weight.

5. Estimates of extracellular water in the muscles (22.8% of total muscle water) have been used for calculations of intracellular concentrations of inorganic ions, phosphates and various non-protein nitrogenous compounds. Two-thirds of the osmotic concentration of the muscle cell is made up by the latter, of which free amino acids constitute only a small fraction.

6. In whole muscle of specimens kept in 20% and 10% sea water the absolute amounts of ions and molecules fall in conformity with blood concentration, the ions increasing as a proportion of the serum concentration from 47% in 100% sea water to 58% and 63%, respectively. In 200% sea water ions in muscle as a proportion of those in serum also rise, to 62%.

LITERATURE CITED

ABEL, O., 1927. *Lebensbilder aus der Tierwelt der Vorzeit* [2nd ed.] Gustav Fischer, Jena, 714 pp.

AYERS, J. C., 1959. The hydrography of Barnstable Harbor, Massachusetts. *Limnol. Oceanogr.,* **4**: 448–462.

Bricteux-Grégoire, S., Gh. Duchâteau-Bosson, Ch. Jeuniaux and M. Florkin, 1962. Constituants osmotiquement actifs des muscles du crabe chinois *Eriocheir sinensis,* adapté à l'eau douce ou à l'eau de mer. *Arch. Int. Physiol. Biochem.,* **70**: 273–286.

Bricteux-Grégoire, S., Gh. Duchâteau-Bosson, Ch. Jeuniaux and M. Florkin, 1966. Les constituants osmotiquement actifs des muscles et leur contribution à la régulation isosmotique intracellulaire chez *Limulus polyphemus. Comp. Biochem. Physiol.,* **19**: 729–736.

Cole, W. H., 1940. The composition of fluids and sera of some marine animals and of the sea water in which they live. *J. Gen. Physiol.,* **23**: 575–584.

Conway, E. J., 1962. *Microdiffusion Analysis and Volumetric Error.* [5th ed.] Crosby Lockwood, London, 467 pp.

Dailey, M. E., F. Fremont-Smith and M. P. Carroll, 1931. The relative composition of sea water and of the blood of *Limulus polyphemus. J. Biol. Chem.,* **93**: 17–24.

Dick, D. A. T., 1959. Osmotic properties of living cells. *Int. Rev. Cytol.,* **8**: 387–448.

Duval, M., 1925. Recherches physico-chimiques sur le milieu intérieur des animaux aquatiques. Modifications sous l'influence du milieu extérieur. *Ann. Inst. Océanogr.,* **2**: 233–407.

Ennor, A. H., 1957. Determination and preparation of N-phosphates of biological origin. II. Phosphoarginine, pp. 857–861. *In:* S. P. Colowick and N. K. Kaplan, Eds., *Methods in Enzymology, Vol. 3.* Academic Press, New York.

Garrey, W. E., 1905. The osmotic pressure of sea water and of the blood of marine animals. *Biol. Bull.,* **8**: 257–270.

Heagy, F. C., 1948. The use of polyvinyl alcohol in the colorimetric determination of magnesium in plasma or serum by means of titan yellow. *Can. J. Res. Series E,* **26**: 295–298.

Howarth, M. K., 1964. The Jurassic period, pp. 203–206. *In:* W. B. Harland, A. G. Smith and B. Wilcock, Ed., *The Phanerozoic Time-Scale.* Geological Society of London, London.

Janus, J. W., 1956. Determination of arginine. *Nature,* **177**: 529.

Krogh, A., 1939. *Osmotic Regulation in Aquatic Animals.* University Press, Cambridge, 242 pp.

Lochhead, J. H., 1950. *Xiphosura polyphemus,* pp. 360–381. *In:* F. A. Brown, Ed., *Selected Invertebrate Types.* Wiley, New York.

Macallum, A. B., 1910. The inorganic composition of the blood in vertebrates and invertebrates, and its origin. *Proc. Roy. Soc. London Series B,* **82**: 602–624.

McManus, J. J., 1969. Osmotic relations in the horseshoe crab, *Limulus polyphemus. Amer. Midland Natur.,* **81**: 569-573.

Pearse, A. S., 1928. On the ability of certain marine invertebrates to live in diluted sea water. *Biol. Bull.,* **54**: 405–409.

Robertson, J. D., 1949. Ionic regulation in some invertebrates. *J. Exp. Biol.,* **26**: 182–200.

Robertson, J. D., 1953. Further studies on ionic regulation in marine invertebrates. *J. Exp. Biol.,* **30**: 277–296.

Robertson, J. D., 1960a. Studies on the chemical composition of muscle tissue. I. The muscles of the hagfish *Myxine glutinosa* L. and the Roman eel *Muraena glutinosa* L. *J. Exp. Biol.,* **37**: 879-888.

Robertson, J. D., 1960b. Ionic regulation in the crab *Carcinus maenas* (L.) in relation to the moulting cycle. *Comp. Biochem. Physiol.,* **1**: 183–212.

Robertson, J. D., 1961. Studies on the chemical composition of muscle tissue. II. The abdominal flexor muscles of the lobster *Nephrops norvegicus* (L.). *J. Exp. Biol.,* **38**: 707–728.

Robertson, J. D., 1965. Studies on the chemical composition of muscle tissue. III. The mantle muscle of cephalopod molluscs. *J. Exp. Biol.,* **42**: 153–175.

Robinson, R. A., 1954. The vapour pressure and osmotic equivalence of sea water. *J. Mar. Biol. Ass. U. K.,* **33**: 449–455.

Robinson, R. A., and R. H. Stokes, 1959. *Electrolyte Solutions.* Butterworths, London, 571 pp.

Roe, J. H., J. H. Epstein and N. P. Goldstein, 1949. A photometric method for the determination of inulin in plasma and urine. *J. Biol. Chem.,* **178**: 839-845.

SCATCHARD, G., AND S. S. PRENTISS, 1933. The freezing points of aqueous solutions. IV. Potassium, sodium and lithium chlorides and bromides. *J. Amer. Chem. Soc.*, **55**: 4355–4362.

SHAW, J., 1958. Osmoregulation in the muscle fibres of *Carcinus maenas*. *J. Exp. Biol.*, **35**: 920–929.

SHUSTER, CARL N., JR., 1950. Observations on the natural history of the American horseshoe crab, *Limulus polyphemus*. *Third report on investigations of methods of improving the shellfish resources of Massachusetts.* Contribution No. 564, Woods Hole Oceanographic Institution, Dec. 31, 1950.

SHUSTER, CARL N., JR., 1957. Xiphosura (with especial reference to *Limulus polyphemus*). *Geol. Soc. Amer., Mem.* **67**(1), 1171–1173.

SHUSTER, CARL N., JR., 1960. Xiphosura. *In: Encyclopedia of Science and Technology* [McGraw-Hill Book Co., Inc.] **14**: 563–567.

SMITH, R. I., 1967. Osmotic regulation and adaptive reduction of water-permeability in a brackish-water crab, *Rhithropanopeus harrisi* (Brachyura, Xanthidae). *Biol. Bull.*, **133**: 643–658.

STØRMER, L., 1952. Phylogeny and taxonomy of fossil horseshoe crabs. *J. Paleontol.*, **26**: 630–640.

SVERDRUP, H. U., M. W. JOHNSON AND R. A. FLEMING, 1942. *The Oceans.* Prentice Hall, New York, 1087 pp.

TAMAR, H., 1965. Absence of hyperglycemic response at or after ecdysis in *Xiphosura polyphemus*. *Biol. Bull.*, **129**: 426–427.

THEEDE, H., 1969. Einige neue Aspekte bei der Osmoregulation von *Carcinus maenas*. [Some new aspects in the osmoregulation of *Carcinus maenas*]. *Marine Biology*, **2**: 114–120.

Reference: *Biol. Bull.*, 138: 184–193. (April, 1970)

NUCLEAR-CYTOPLASMIC RELATIONS IN THE MITOSIS OF SEA URCHIN EGGS. II. THE DIVISION TIMES OF WHOLE EGGS AND HAPLOID AND DIPLOID HALF-EGGS[1]

RONALD C. RUSTAD, SHUHEI YUYAMA,[2, 3] AND LYNNE C. RUSTAD[3, 4]

Departments of Radiology and Biology, Case Western Reserve University,
Cleveland, Ohio 44106

The relationship of the duration of the first mitotic cycle of marine eggs to the ploidy and the cytoplasmic volume can be studied by fertilizing nucleate and anucleate egg fragments. Such fragments can be prepared by shaking (Hertwig and Hertwig, 1887), drawing newly-fertilized eggs rapidly through a narrow orifice (C. B. Metz, personal communication), centrifugation (Lyon, 1907, *cf.* Harvey, 1956), and microsurgery (Delage, 1898). The first two mechanical methods produce egg fragments of various sizes and also produce varying amounts of cellular debris depending on the vigor of the treatment. Tennent (1912) stated that cytoplasmic fragments prepared by shaking and subsequently fertilized cleaved with the same "tempo" as the whole eggs. Although normal plutei can be raised from anucleate fragments prepared by the shaking method, the possibilities of cell damage and even of undetected nuclear fragments led Boveri (1918) to question the usefulness of this technique for embryological experiments. The first postfertilization division of enucleated half and quarter sea urchin eggs prepared by centrifugation is considerably retarded as compared to the whole egg or nucleate half; however, the nucleate quarter-egg is also greatly delayed in its division (*e.g.*, Harvey, 1932, 1956). Since the centrifugation method stratifies cytoplasmic organelles, the results obtained are to some extent a reflection of the cytoplasmic composition of the different types of fragments (*cf.* Moore, 1938; Kojima, 1959).

Variable results have been reported by different workers who prepared egg fragments by cutting with microneedles. In general, the conclusions have been based on very small numbers of eggs, and the possibility of surgical injury has not always been ruled out. In an earlier study (Rustad and Rustad, 1960) we found that amputating approximately half of the cytoplasm after fertilization had no obvious effect on the division time and that nucleate fragments as small as onethirtieth of the whole cell volume could be fertilized and would divide at approximately the same time as the whole eggs.

---

[1] Supported by Contract W-31-109-ENG (Report Number COO-78-184) with the U. S. Atomic Energy Commission. The experiments with *Arbacia* were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts. The techniques employed in this study were developed with the support of the U. S. Atomic Energy Commission while the authors were affiliated with the Department of Biological Sciences and the Institute of Molecular Biophysics of Florida State University, Tallahassee, Florida.

[2] Present address: Department of Zoology, University of Toronto, Toronto, Canada.

[3] Supported in part by contract Nonr 4785(00) between the Office of Naval Research and the Marine Biological Laboratory, Woods Hole, Massachusetts.

[4] Present address: Department of Psychology, Case Western Reserve University, Cleveland, Ohio 44106.

With improved techniques it has been possible to prepare adequate numbers of nearly equal-sized nucleate and anucleate half-eggs for a careful comparison of the division times of whole and haploid and diploid half-eggs. Thus, it has been possible to determine whether or not either ploidy or cytoplasmic volume influence the duration of the mitotic cycle.

Materials and Methods

Gametes were obtained from the sea urchin *Arbacia punctulata* (collected at Woods Hole, Massachusetts) by stimulating the animals with a 12 volt alternating current (Iwata, 1950; Harvey, 1953). *Lytechinus pictus* (shipped by air to Cleveland, Ohio, from the Pacific Biomarine Supply Co., P.O. Box 285, Venice, California 90293) were induced to shed gametes by injecting them with 0.55 *M* KCl (Tyler, 1949). The eggs from *Arbacia* were washed three times in filtered sea water; the eggs from *Lytechinus* were washed three times in "Instant Ocean" (Aquarium Systems, Inc., 1450 E. 289 Street, Wickliffe, Ohio). Agar (0.2 to 0.3% in 80% sea water or in 80% "Instant Ocean") was heated and poured into Syracuse watch glasses. After the agar gelled the dishes were filled with sea water or "Instant Ocean" and washed in it several times while being stored for at least 24 hours in a refrigerator. This extensive washing procedure appeared to be necessary in order to prevent fragility, crenation, and even partial activation of the eggs by soluble materials in the agar gel. If the agar was too soft, the eggs would sink into it during cutting, and if it was too hard, the eggs tended to roll out from under the microneedle. The microneedles were prepared freehand over a microburner from thin-walled 2-mm glass capillary tubing. A concentrated suspension of eggs was placed in the center of an agar-coated Syracuse dish and the eggs were cut freehand into "halves" which appeared equal in size when examined with a Zeiss or Wild dissecting microscope. The cutting procedure consisted of resting the tip of the microneedle on the agar at some distance from the egg and then pressing the needle slowly down (often with a slight sawing motion) until the cell membrane on each side of the egg fused with itself and the two halves separated. Eggs from some animals flattened considerably under the pressure of the microneedle, so it was possible to make the cut through the widest part of the cell and obtain approximately equal-sized "halves." Other eggs tended to flow upward beside the needle so that a continuous adjustment of needle position was needed in order to produce two spheres of apparently equal diameter. Eggs which had not lost most of their jelly coat during the washing procedures were usually difficult to cut. If the eggs from a particular animal were fragile or difficult to cut, eggs from another animal were used. Since the nuclei of unflattened eggs of *Arbacia* are sometimes difficult to see under a dissecting microscope, the nucleate and anucleate halves were usually separated immediately after cutting. Nuclei in eggs of *Lytechinus* were always easy to see, so the half-eggs could be separated later. The half-eggs were often more difficult to fertilize yet more susceptible to polyspermy than whole ones. Therefore, considerable caution had to be exercised in fertilizing. The number of eggs of each type with division furrows were counted during the division period.

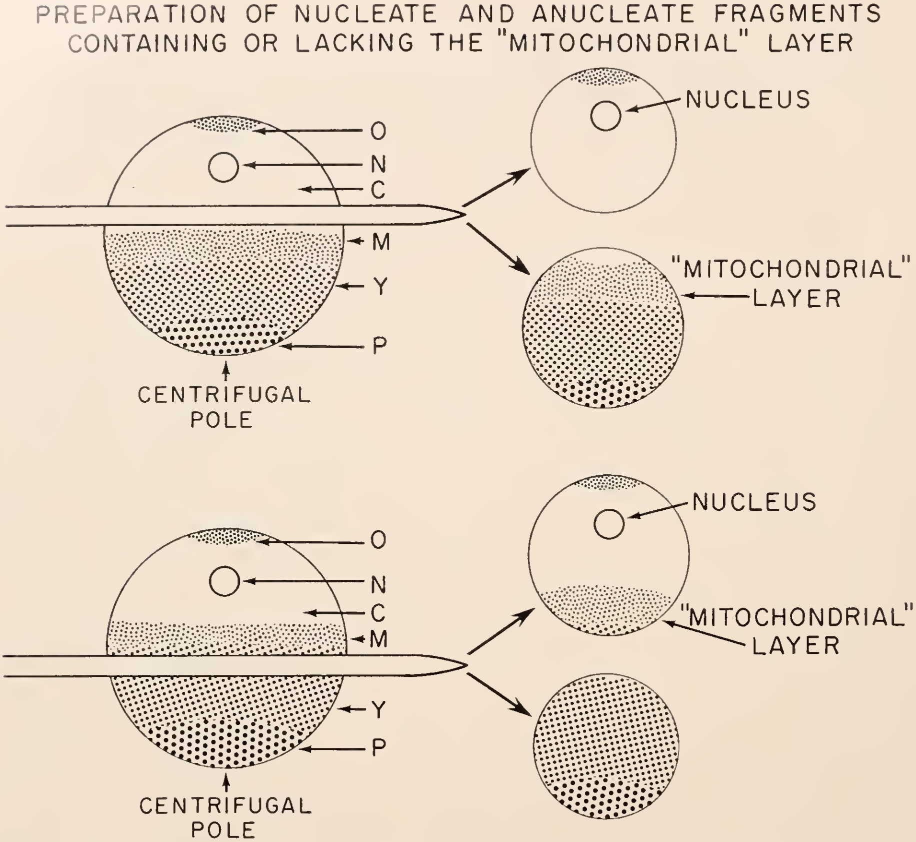


FIGURE 1. Microsurgery of eggs which have been stratified by centrifugation. Nucleate fragments lacking or containing the "mitochondrial layer" are formed when the microneedle passes on the centripetal or centrifugal side of the layer, respectively; Oil cap (O); Nucleus (N); Clear cytoplasm (C); "Mitochrondrial layer" (M); Yolk Granules (Y); Pigment granules (P).

In some experiments quarter-eggs were prepared by cutting nucleate or anucleate half-eggs into halves. In a limited number of experiments with *Arbacia* the cytoplasmic organelles of the eggs were stratified by centrifuging the cells at the interface between sea water and 1.1 *M* sucrose for 10 minutes at 10,000 times gravity (see Harvey, 1956). The eggs were quickly removed from the centrifuge tubes and mixed with sea water in the cutting dishes. These eggs were cut either on the centripetal or centrifugal side of the visible "mitochrondrial layer" before any redistribution of the stratified organelles could be detected (Fig. 1). Thus, both nucleate and anucleate cells either containing or lacking the "mitochondrial layer" were produced.

RESULTS

Whole eggs and diploid half-sized eggs divided at the same time while haploid half-sized eggs divided later. The distributions of division times of individual eggs in a typical experiment are shown in Figure 2. In some experiments the number of late-dividing cells was somewhat larger in samples of diploid halves than of wholes; however, the times when 50% of the cells had cleaved did not appear to differ significantly.

The differences in times of 50% division of individual samples of more than 30 eggs or half-eggs (obtained from curves such as figure 2) have been analyzed. The data were derived from 796 diploid half-eggs in 25 separate experiments. The calculated average division time of the diploid half-egg is 1.3 ± 1.5 minutes earlier than the whole cell in the case of *Arbacia* (16 experiments with a total of 646 whole and 544 half-eggs) and 1.2 ± 0.8 minutes later than the whole cell in the case of *Lytechinus* (9 experiments with a total of 330 whole and 252 half-eggs). Since the usual error of estimating the time of 50% cleavage of very large populations from single animals is approximately ± 1 minute, (*cf.* Harvey, 1956) these average differences cannot be considered significant. If the data from all of the experiments are averaged without regard to the genus of eggs, the calculated modification of division caused by removing half of the cytoplasm would not exceed one half minute (approximately 1% of the total duration of the mitotic cycle). Thus, amputation of half of the cytoplasm does not cause a significant mitotic delay.

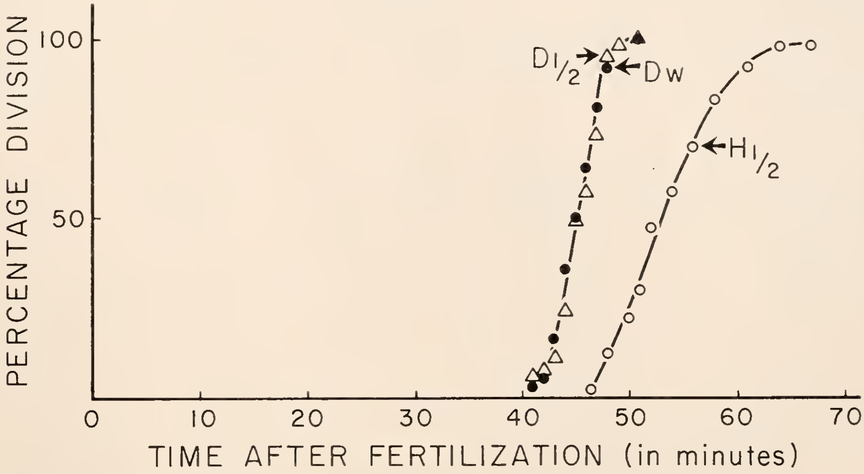


FIGURE 2. First division times of diploid whole eggs and of diploid and haploid half-eggs; Diploid whole eggs (D_W, solid circles); Diploid half-eggs ($D_{\frac{1}{2}}$, open triangles); Haploid half-eggs ($H_{\frac{1}{2}}$, open circles).

The haploids divided later than either the whole cells or the diploid halves in every experiment (*e.g.,* Fig. 2). The delay of the time when 50% of the haploids cleaved was variable, ranging from 5 to 15 minutes. There did not appear to be any systematic correlation between the amount of delay and the genus of egg or period of the breeding season.

Only a limited number of fragments could be prepared from centrifuged eggs in any experiment, because it is difficult to control the plane of cutting and the stratified organelles became redistributed rapidly by Brownian motion. When the cut was made on the centripetal side of the "mitochondrial layer" (Fig. 1), the nucleate fragments could be fertilized (elevating fertilization membranes) but did not divide within 24 hours. However, when the "mitochondrial layer" was included in the nucleate fragments (Fig. 1) they divided at approximately the same time as whole eggs. When anucleate eggs which lacked the "mitochondrial layer" were fertilized they divided some 40 to 70 minutes later than whole eggs. In contrast, haploid fragments which contained the "mitochondrial layer" were only delayed from 5 to 10 minutes compared to whole cells and were not detectably delayed compared to uncentrifuged haploids from the same animal. Thus, some factor found in the "mitochondrial layer" appears to be essential for a normal rate of division.

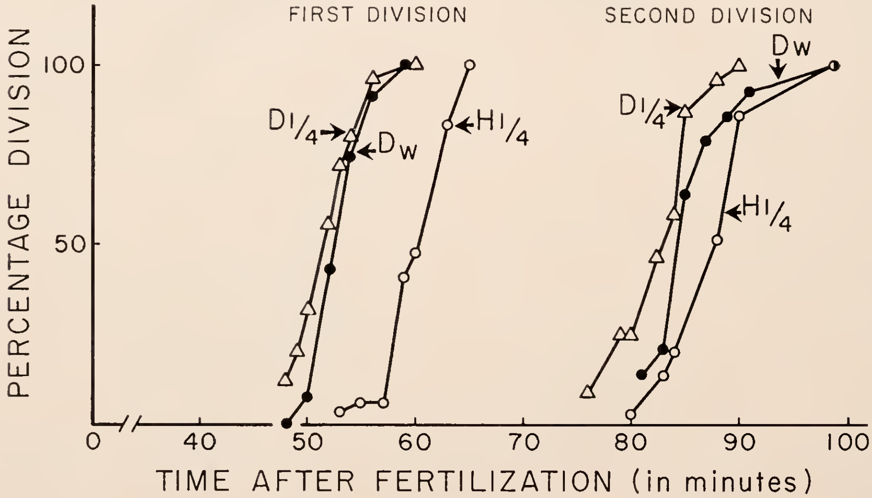


FIGURE 3. First and second division times of diploid whole eggs and of diploid and haploid quarter-eggs; Diploid whole eggs (D_W, closed circles); Diploid quarter-eggs ($D_{1/4}$, open triangles); Haploid quarter eggs ($H_{1/4}$, open circles).

Neither ploidy nor cell volume appeared to influence the duration of the second mitotic cycle. An extreme test involved comparing diploid whole cells with haploid and diploid quarter-sized eggs. The times of 50% first divisions of the whole eggs and the diploid quarter-sized cells did not differ by more than one minute, while the haploid quarter was delayed 7 minutes (Fig. 3, Table I). The durations of the second mitotic cycles of the whole embryos and the diploid quarter-sized ones were identical. The three-minute difference in the duration of the second cycle measured for the haploid quarter-sized embryos is not significant (Fig. 3, Table I). Hence, the duration of the second mitotic cycle is not influenced by a twofold variation in the ploidy or by a fourfold variation in the cytoplasmic volume.

Additional observations on the time of the fourth mitosis of whole, and both haploid and diploid quarter-eggs indicated that size had no obvious cumulative effect on the time of the fourth mitosis. Similarly, in haploid half-sized embryos the total increase in the interval between fertilization and the fourth mitosis was equivalent to the delay of the first division rather than to an accumulation of four successive delays of similar magnitude.

The data indicate that cell volume does not influence the duration of the early mitotic cycles of the sea urchin egg and that even though haploid cells are delayed in their first mitosis, the subsequent cell cycles are not significantly retarded.

Discussion

Diploid whole and half-eggs

The present data on 796 half-eggs demonstrated that there is no significant difference between the times of division of 50% of a population of whole sea urchin eggs or 50% of a population of half-sized diploid eggs. Hence, the volume of cytoplasm does not affect the duration of the mitotic cycle of eggs of either *Arbacia punctulata* or *Lytechinus pictus.*

Table I

Division times of whole and quarter-eggs following fertilization

	Time of first division	Time of second division	Length of second division cycle
Diploid Whole	52 min	84 min	32 min
Diploid Quarter	51	83	32
Haploid Quarter	59	88	29

Previous workers using micromanipulators or cutting freehand were not able to obtain many half-eggs. For instance, Whitaker's (1929) conclusion that the diploid half-egg divided slightly sooner than whole cells depends on a total of 43 uncentrifuged diploid half-eggs cut during the course of 8 separate experiments, even though cells cut after centrifugation yielded similar data. The conflicting conclusions from other early investigations employing microsurgery were based on even smaller numbers of cells in each experiment (see Table II). Therefore, it seems that the conclusions of all previous workers were drawn from inadequate data.

Haploid versus *diploid eggs*

In each of 25 experiments in the present study involving more than 782 of each type of half-egg, the haploid half-eggs divided later than the wholes and diploid halves. This result was also obtained by some of the earlier workers who cut sea urchin eggs (see Table II) and may have been valid even when their measurements of smaller apparent differences between whole cells and diploid halves could be ascribed to sampling errors.

Although no data were presented, Tennent (1912) stated that haploid fragments obtained by shaking eggs of *Toxopneustes* (*Lytechinus*) had the same "tempo" of cleavage as diploid eggs. The classical crossfertilization experiments of Moore (1933) suggest in a very general way that the *type* of cytoplasm can limit the rate of cleavage. Eggs from *Strongylocentrotus* fertilized with sperm from the sand

TABLE II

A comparison of some previous studies concerning the relationship of ploidy and cytoplasmic volume to the cleavage time of echinoderm eggs

Method	Worker	Conclusion	Genus	Remarks
		Regarding duration of division cycle*		
Cut	Delage (1898)	$D_W < D_{\frac{1}{2}} < H_{\frac{1}{2}}$	*Strongylocentrotus*	12 haploid eggs and no quantitative data presented.
Shake	Tennent (1912)	$D_W = D_{\frac{1}{2}} = H_{\frac{1}{2}}$	"*Toxopneustus*" (*Lytechinus*)	No quantitative data presented.
Cut	Tennent, Taylor and Whitaker (1929)	$D_W < D_{\frac{1}{2}} < H_{\frac{1}{2}}$	*Lytechinus*	Many eggs cut, but only about 45% cleaved. Daily data on cleavage time of whole eggs were not reported.
Cut	Whitaker (1929)	$D_{\frac{1}{2}} < D_W < H_{\frac{1}{2}}$	*Arbacia*	43 haploid eggs. (Same conclusion with cut-centrifuged eggs):
Centrifuged	Harvey (1932)	$D_W \cong D_{\frac{1}{2}} \ll H_{\frac{1}{2}}$	*Arbacia*	Centripetal diploid quarters even slower. Furrows do not form on haploid half until many nuclei are present.
Cut	Moore (1933)	$D_W \cong D_{\frac{1}{2}} \cong H_{\frac{1}{2}}$	a) *Dendraster* ♂ x ♀ b) *Strongylocentrotus* ♂ x *Dendraster* ♀	One haploid sand dollar egg- 3 haploid eggs. Sea urchin-sand dollar "hybrids."

* D_W = Diploid whole egg; $D_{\frac{1}{2}}$ = Diploid half-egg; $H_{\frac{1}{2}}$ = Haploid half-egg.

dollar *Dendraster* divided at about the same time as normal eggs from *Strongylocentrotus* (*ca.* 95 min), and similarly, eggs from *Dendraster* fertilized with sperm from *Strongylocentrotus* cleaved at approximately the same time as the normal sand dollar eggs (*ca.* 55 min). These experiments with whole eggs suggest that the control of division time is either by the female pronucleus or by the cytoplasm, but not by the male pronucleus from the other order of echinoderm. Moore (1933) also fertilized four enucleated eggs fragments but the data seem insufficient to draw any conclusions concerning the normal nuclear-cytoplasmic relations in the timing of mitosis (see Table II). Thus, there appear to be no adequate data on any genus to contradict our general conclusion that first division of haploid half-eggs occurs later than that of either whole cells or diploid half-cells.

Centrifuged eggs

Nucleate fragments were prepared by cutting on the centripetal and centrifugal sides of the visible "mitochondrial layer" of the stratified eggs. The nucleate cells without the "mitochondrial layer" elevated fertilization membranes but did not divide, while those containing it divided at approximately the same time as whole eggs. This result agrees with results of studies in which half- and quarter-eggs were prepared by centrifuging until half- and/or quarter-eggs were pinched off as a result of density differences within the stratified eggs (reviewed by Harvey, 1956; see also: Moore, 1938; Kojima, 1959). Diploid half-eggs (from the centripetal half) divide relatively normally but smaller fragments which do not contain enough of some components found in or near the "mitochondrial layer" are either delayed many hours in their division or are unable to divide.

The division of haploid cells lacking the "mitochondrial layer" was found to be considerably retarded, as is also known to be true of such halves prepared directly by centrifugation (*e.g.*, Harvey, 1956). However, when anucleate fragments containing the "mitochondrial layer" were fertilized the division was only slightly retarded compared to whole eggs and no distinction could be made between the division times of these haploid cells and ones which had been prepared from uncentrifuged cells. Since removing the "mitochondrial layer" merely delayed the division of haploid centrifugal cells (rather than blocking it as in the case of the diploid centripetal cells) it would appear that some substance which is essential for division is found in the "mitochondrial layer" and is also present in a limited quantity in the more centrifugal parts of the cell. Moore (1938) studied the removal of a "cleavage substance" from the centripetal half of sand dollar eggs by centrifugation. Kojima (1959) suggested a correlation between the ability or inability of diploid fragments to divide, and the presence or absence of granules, which could be vitally stained with neutral red (as lysosomes are in some cells). Mitochondria are found throughout centrifuged eggs; however, there is a preferential accumulation of mitochondria with a clear matrix in the centrifugal half and those with a dense matrix in the centripetal half (Geuskens, 1965).

The postfertilization respiration rate of the haploid centrifugal fragment is only about half as great as that of the diploid centripetal fragment containing the "mitochondrial layer" (Shapiro, 1935). The present experiments clearly demonstrate that the "mitochondrial layer" contains some substance required for division which is not present in the more centrifugal parts of the cell in adequate abundance to permit a normal division rate. However, we do not know whether this "cleavage substance" is found in lysosomes, mitochondria or some unidentified structure.

Later divisions

The fact that the normal durations of the second, third, and fourth mitotic cycles are quite similar (*e.g.*, Harvey, 1956) indicates that the division rate is not strongly dependent upon cytoplasmic volume. Although the first mitosis occurred later in haploid than in diploid half-eggs in the present experiments, there was no comparable relay in the next three mitoses. The duration of the second mitotic cycle was not significantly influenced by either ploidy or by a fourfold reduction of cytoplasmic volume. It was not possible to measure successively each of the later division times with the same precision as the first and second. However, if the second, third, and fourth mitotic cycles of the haploid cells had been delayed as much as the first, the cumulative delay by the time of the fourth division would have been 20 to 60 minutes. Delays of such magnitude were never encountered. Therefore, notwithstanding a delay of the first division of haploid cells, the amount of nuclear genetic material *per se* does not seem to limit the rate of mitosis. The delay of the first mitosis of a half-egg containing only a male pronucleus appears to be associated with the initiation of some activity associated with the first nuclear division cycle. Since such activities as protein synthesis can be activated artificially in anucleate eggs (reviewed by Tyler, 1966), it seems unlikely that the female pronucleus *per se* controls the initiation of cytoplasmic activities. It seems more probable that the division delay occurs in the initiation of, or the early events of the nuclear replication cycle. The DNA of the male pronucleus may remain compactly oriented and unable to replicate for a longer time when fusion with the larger more diffuse female pronucleus does not occur. Alternatively, the female pronucleus may contain enzymes and/or precursors for chromosomal duplication. Whatever factors limit the rate of the first but not later mitoses of the haploid egg, neither cytoplasmic volume nor ploidy appears to have any significant role in regulating the rate of cell division of sea urchin eggs.

SUMMARY

Sea urchin eggs were cut into nucleate and anucleate halves. Following fertilization, the diploid half- and quarter-cells divided at the same time as whole cells, and the haploid half- and quarter-cells divided significantly later. However, there was no comparable increase in the duration of the second, third and fourth mitotic cycles of the haploid cells. Hence, the delay of the first mitosis of the haploid half-cells was attributed to the special consequences of the male pronucleus not fusing with a female pronucleus, and it was concluded that neither the cytoplasmic volume nor the ploidy of sea urchin eggs have significant effects on the timing of the mitotic cycle. Cutting experiments on eggs whose cytoplasmic organelles had been stratified by centrifugation suggested that some factor concentrated in the "mitochondrial layer" is essential for the maintenance of normal mitotic rates.

LITERATURE CITED

BOVERI, T., 1918. Zwei Fehlerquellen bei Merogonieversuchen und die Entwicklungsfähigkeit merogonischer und partielmerogonischer Seeigelbastarde. *Wilhelm Roux Arch. Entwicklungsmech. Organismen.*, **44**: 417–471.

DELAGE, Y., 1898. Embryons sans noyau maternel. *C. R. Seances Acad. Sci.*, Paris, **127**: 528–531.

GEUSKENS, M., 1965. A study of the ultrastructure of nucleate and anucleate fragments of unfertilized sea urchin eggs. *Exp. Cell Res.*, **39**: 413–417.

HARVEY, E. B., 1932. The development of half and quarter eggs of *Arbacia punctulata* and of strongly centrifuged whole eggs. *Biol. Bull.*, **62**: 155–167.

HARVEY, E. B., 1953. A simplified electrical method for determining the sex of sea urchins and other marine animals. *Biol. Bull.*, **105**: 365.

HARVEY, E. B., 1956. *The American Arbacia and Other Sea Urchins.* Princeton University Press, Princeton, New Jersey, 298 pp.

HERTWIG, R., AND O. HERTWIG, 1887. Über die Befruchtungs- und Teilungsvorgange des tierischen Eis unter dem Einfluss auserer Agentien. *Z. Med. Naturwiss., Jena,* **20**: 107–262.

IWATA, K. S., 1950. A method of determining the sex of sea urchins and of obtaining eggs by electric stimulation. *Annot. Zool. Jap.* **23**: 39–42.

KOJIMA, M. K., 1959. Relation between vitally-stained granules and cleavage activity in the sea urchin egg. *Embryologia,* **4**:191–209.

LYON, E. P., 1907. Results of centrifugalizing eggs. *Wilhelm Roux Arch. Entwicklungsmech. Organismen.,* **23**: 151–173.

MOORE, A. R., 1933. Is cleavage rate a function of the cytoplasm or of the nucleus? *J. Exp. Biol.,* **10**: 230–236.

MOORE, A. R., 1938. Segregation of "cleavage substance" in the unfertilized egg of *Dendraster excentricus. Proc. Soc. Exp. Biol. Med.,* **22**: 305–306.

RUSTAD, R. C., AND L. C. RUSTAD, 1960. Nuclear-cytoplasmic relations in the mitosis of sea urchin eggs. *Ann. N. Y. Acad. Sci.,* **90**: 531–535.

SHAPIRO, H., 1935. The respiration of fragments obtained by centrifugating the egg of the sea urchin, *Arbacia punctulata. J. Cell. Comp. Physiol.,* **6**: 101–116.

TENNENT, D. H., 1912. Studies in Cytology I, A further study of the chromosomes of *Toxopneustes variegatus. J. Exp. Zool.,* **12**: 391–411.

TENNENT, D. H., C. V. TAYLOR AND D. M. WHITAKER, 1929. An investigation on organization in a sea urchin egg. *Carnegie Inst. Washington, Pub., No.* **391**: 1–104.

TYLER, A., 1949. A simple non-injurious method for inducing repeated spawning in sea urchins and sand dollars. *Collecting Net,* **19**: 6–8.

TYLER, A., 1966. Incorporation of amino acids into protein by artificially activated non-nucleate fragments of sea urchin eggs. *Biol. Bull.,* **130**: 450–461.

WHITAKER, D. M., 1929. Cleavage rates in fragments of centrifugated *Arbacia* eggs. *Biol. Bull.,* **57**: 159–171.

Reference: *Biol. Bull.*, **138**: 194–199. (April, 1970)

THE EFFECT OF CESIUM-137 GAMMA RAYS ON REGENERATION IN *TUBULARIA*[1]

MARGARET SLAUGHTER, FLORENCE C. ROSE AND ANTHONY LIUZZI

Department of Epidemiology and Public Health, Yale University, School of Medicine, New Haven, Connecticut 06510; Department of Anatomy, Tulane University, New Orleans, Louisiana 70037, and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Inhibition of regeneration by irradiation was first observed by Bardeen and Baetjer (1904). Using an x-ray tube they demonstrated that Roentgen rays had an inhibitive effect upon cell-reproduction in planarians..

Years later, Curtis and Ritter (1927) demonstrated that x-rays inhibited regeneration in *Tubularia crocea,* but a definite radiation exposure was not determined. Inhibition of regeneration by x-irradiation was also observed in the limbs of *Amblystoma* (Butler, 1931). Puckett (1936) reported complete inhibition of regeneration in colonies of *Pennaria tiarella* exposed to 10,500 R or more. Lower exposures retarded the regeneration of hydranths, but failed to check the process completely. These early experiments demonstrated that x-irradiation markedly effects regeneration.

The initial objective of this study was to determine a specific non-lethal exposure of ionizing radiation that would inhibit regeneration in *Tubularia.* Previous investigations have shown that coelenterates are highly radiation resistant, but no specific information was available to verify this fact for the marine forms.

This paper describes the response of *Tubularia* to cesium-137 irradiation. Studies were made over a wide range of exposures including lethal levels. General changes in the regeneration process were observed and characterized.

MATERIALS AND METHODS

The forms of *Tubularia* used in these experiments grow in the Woods Hole, Massachusetts area throughout the summer. Only fresh material brought to the laboratory daily was used.

To induce the process of regeneration, stems 5 mm to 10 mm in length were cut from the colonies. A transverse cut was made below the hydranth and oblique cut 5 mm to 10 mm below the transverse cut. The transverse and oblique cuts were made to distinguish between distal and proximal ends of the cut stems. Cut stems were kept in glass finger bowls covered with cheese cloth and placed under running sand-filtered sea water.

Stems were irradiated using 667 Kev gamma rays from a 5000 Curie cesium-137 source at an exposure rate of 4000 R/min. All stems were irradiated in a Petri dish containing sea water and transferred to finger bowls postirradiation. Except for pilot experiments, all irradiations were completed within two hours after cutting.

---

[1] This work was supported in part by the U. S. Public Health Service Grant RH-70-01S469.

Exposures ranged from 10,000 R to 350,000 R requiring 2.5 to 87.5 minutes of irradiation.

The temperature during irradiation ranged from 10° C to 23° C. This was dependent upon the time required to accumulate a particular radiation exposure. For exposures greater than 75,000 R, iced sea water varying from 10° C to 18° C was used and changed during the irradiation procedure. Earlier observations showed that a temperature rise above 23° C during irradiation killed the organism.

Stems were observed under a dissecting microscope at different time periods post-irradiation. Seven stages of regeneration were defined. These were: pink, striated, double striated, pinched, bundle, emerging and emerged (Fig. 1). At each observation time a count was made of stems in each category. Stems undergoing no regeneration were also counted. Unirradiated stems fully regenerated complete hydranths within 36 to 48 hours after cutting. All stems died when kept more than five days in this particular laboratory situation.

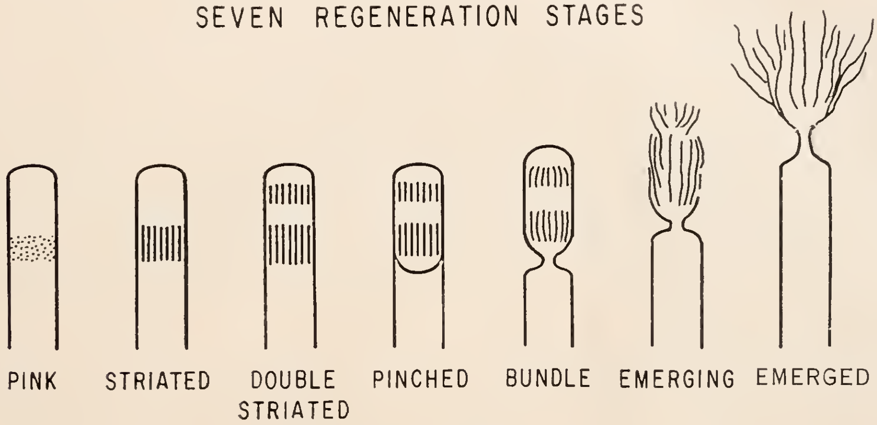


Figure 1. A diagrammatic representation of seven recognizable stages in *Tubularia* regeneration.

Results

Time of irradiation

Pilot experiments were performed to establish an optimum irradiation procedure. Preliminary experiments showed that stems of *Tubularia* will undergo complete regeneration at a radiation exposure of 10,000 R, but at a slower rate when compared with unirradiated stems. Experiments were performed to determine the most effective time to irradiate the organism. Groups of 10 stems were cut within the same time interval and exposed to 10,000 R at 2, 6, 12 and 18 hours after cutting. Comparisons were made of the number of stems from each group in the emerged stage of regeneration at these times. No stems, controls or irradiated, had reached the emerged stage 24 hours aftr cutting. Forty-two hours after cutting 0, 6, 3 and 9 stems irradiated, respectively, 2, 6, 12 and 18 hours

TABLE I

Accumulated delay in regeneration at various radiation exposure levels

# of stems in each group	Exposure	Time (hours)									
		30	36	42	48	54	60	66	72	78	90
		Per cent of stems pinched and beyond at various observation times after cutting									
20	Controls	25	55	85	80	95	100	100	100	100	100
20	10,000 R	10	40	60	80	85	90	90	85	80	80
20	20,000 R	5	30	40	50	65	80	90	85	85	85
20	50,000 R	0	5	5	30	30	40	65	65	70	70
20	75,000 R	0	0	0	0	5	10	20	45	45	65
20	100,000 R	0	5	5	5	15	20	25	25	40	40

after cutting had emerged. In addition, a group of 10 stems were irradiated at 10,000 R immediately after cutting; when observed 44 hours after cutting, only one stem in this group had emerged. Based on these data, it was concluded that maximum effectiveness is achieved if stems are irradiated within two hours after cutting. The general results presented below were based on subsequent experiments in which irradiation of stems was completed within two hours after cutting.

General radiation effects

Stems irradiated at 10,000 R and 20,000 R progressed through all the stages of regeneration, but the emerged hydranths had shorter tentacles than those hydranths regenerated from un-irradiated stems. This shortening of tentacle length became more pronounced as the radiation exposure increased.

At exposures of 75,000 R and 100,000 R no tentacles developed on those hydranths that formed, even though the hypostome emerged from the perisarc. Characteristic stages of *Tubularia* regeneration are identified by striations developing at the regenerating sites. The striations are developing distal and proximal

TABLE II

Delay in regeneration at various radiation exposure levels

# of stems in each group	Exposure	Time (hours)									
		20	36	42	48	54	60	66	72	78	90
		Per cent of stems emerged at various observation times after cutting									
20	Controls	0	1	35	50	80	90	100	100	100	100
20	10,000 R	0	0	5	35	55	90	90*	90	90	90
20	20,000 R	0	0	0	15	35	65	70	90*	90	90
20	50,000 R	0	0	0	0	20	25	50	40	65	70
20	75,000 R	0	0	0	0	0	5	15	25	40	65
20	100,000 R	0	0	0	0	5	10	20	25	35	40

* Indicates that at later observations emerged hydranths had begun to drop off.

tentacles. The lack of tentacle growth at high exposures made the various stages of regeneration less recognizable.

Observations and comparisons of regenerated hydranths showed that hydranths regenerated from irradiated stems tended to drop off sooner than those hydranths regenerated from un-irradiated stems.

Stems were exposed to 50,000; 75,000; 100,000; 150,000 and 200,000 R to determine an exposure that completely inhibits regeneration. Observations of the stems after irradiation indicated that 150,000 R and 200,000 R inhibit regeneration, but were not lethal to the organism 90 hours after cutting.

The inhibitory effect of irradiation appeared to be dose dependent. As the exposure increased, the delay in the time of regeneration increased. From recordings of the number of stems in each stage of regeneration for each exposure, all stages appeared to be uniformly affected by irradiation. Table I is a summary of data on delay in regeneration at various radiation exposure levels based on the percentage of stems completing the pinched and all subsequent regeneration stages within the given observation intervals. Table II summarizes data on the observed delay in regeneration time of emerged stems at various exposure levels. In this experiment those irradiated stems that did emerge were, respectively, 6, 12, 18 and 30 hours behind the un-irradiated stems. Figure 2 is a graphic representation of the data in Table II. The percentage of stems completing the emerged stage of regeneration decreases as the exposure levels increase.

Stems were exposed to radiation levels ranging from 225,000 R to 350,000 R in steps of 25,000 R. At an exposure of 300,000 R, 10 out of 20 stems showed

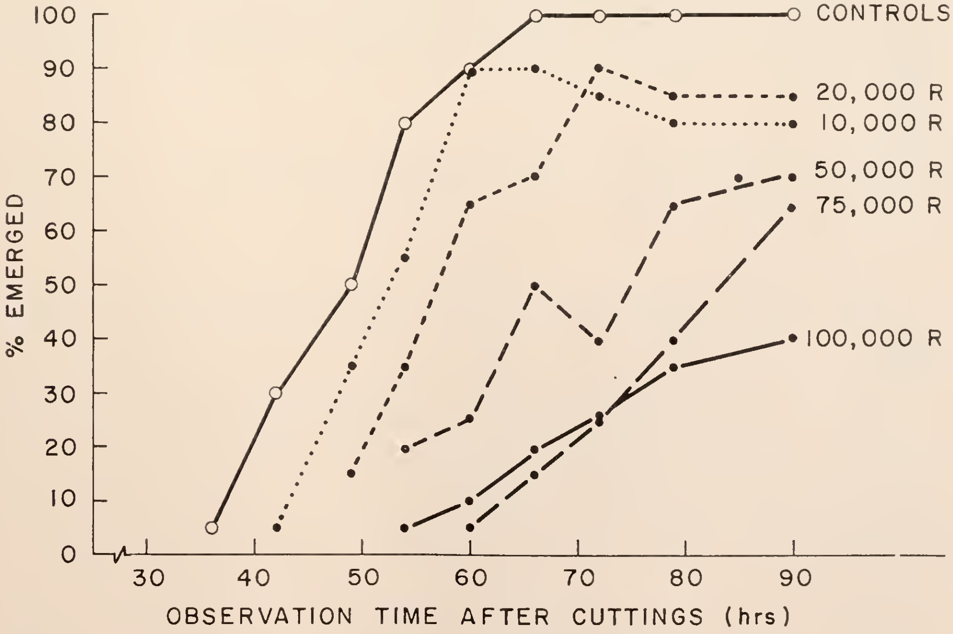


FIGURE 2. A comparison of the percentage of irradiated and control stems in the emerged stage of regeneration at different observation times after cutting.

signs of tissue disintegration when observed six hours after cutting. At eighteen hours after cutting and irradiating all 20 stems were dead. Exposures of 300,000 R and above are fatal to the organism under the given experimental conditions. For the various experiments described a total of 900 organisms was used in ten distinct experiments.

DISCUSSION

It is well-known that ionizing radiation affects regeneration in various invertebrates and vertebrates. Irradiation has frequently been used as a means of studying biological mechanisms involved in the regeneration process. The quantity of radiation necessary to inhibit regeneration is extremely variable. In the coelenterates, various genera react differently; some coelenterates are more radiation resistant that others. X-irradiated hydra whose interstitial cells have been destroyed are still capable of limited regeneration. (Brien and Reniers-Docoen, 1955). Also for the hydra, an exposure of 4500 R inhibits bud formation (Park, 1958). Colonies of *Pennaria tiarella* require 10,500 R to inhibit regeneration. For the colonial hydroid, *Tubularia*, an exposure of 150,000 R is required to inhibit regeneration.

Inhibitory exposures have also been determined in various other organisms. Regeneration in the polychaete, *Clymenella torquata* is inhibited at an exposure of 50,000 R (Rose, F., unpublished observations). X-irradiation ranging from 1000 R to 10,000 R when applied locally will prevent limb and tail regeneration in the adult urodele, *Triturus* (reviewed by Rose, 1964).

The lethal exposure of 300,000 R observed in the present study for *Tubularia* is in the range of exposures required to deactivate many viruses. Viruses lose their infectivity when exposed to radiations ranging from 430,000 R to 5,150,000 R (Luria, 1951).

The authors would like to express their sincere appreciation to Dr. S. Meryl Rose for his advice and interest in this investigation.

SUMMARY

1. Short tentacles developed on hydranths regenerated from stems of *Tubularia* exposed to cesium-137 gamma irradiation at exposure levels of 10,000 R and 20,000 R.

2. Those hydranths that did regenerate from irradiated stems tended to drop off sooner than hydranths regenerated from un-irradiated stems.

3. Exposures of 150,000 R and 200,000 R completely stop regeneration in *Tubularia*; 300,000 R is fatal to the organism.

4. The observed delay in time of regeneration increased as the exposure levels increased and thus appeared to be dose dependent.

LITERATURE CITED

BARDEEN, C. R., AND F. H. BAETJER, 1904. The inhibitive action of the Roentgen rays on regeneration in planarians. *J. Exp. Zool.*, 1: 191–195.

Brien, P., and M. Reniers-Decoen, 1955. Cellules interstitielles des hydres d'eau douce. *Bull. Biol. France Belq.*, **89**: 259–325.

Butler, E. G., 1931. X-radiation and regeneration in *Amblystoma*. *Science,* **74**: 100.

Curtis, W. C., and R. Ritter, 1927. Further studies on the effects of X-radiation on regeneration (abstract). *Anat. Rec.*, **37**: 128.

Luria, S. E., 1951. Radiation and viruses, pp. 333–364. *In:* Alexander Hollaender, Ed., *Radiation Biology, Volume II.* McGraw-Hill- New York.

Park, H. D., 1958. Sensitivity of hydra tissues to X-rays. *Physiol. Zool.*, **31**: 188.

Puckett, W. O., 1936. The effects of X-irradiation on the regeneration of the hydroid, *Pennaria tiarella*. *Biol. Bull.*, **70**: 392–399.

Rose, S. M., 1964. Regeneration, pp. 545–622. *In:* J. A. Moore, Ed., *Physiology of the Amphibia*. Academic Press, New York.

Reference: *Biol. Bull.*, **138**: 200–210. (April, 1970)

DIURNAL MOVEMENTS AND DISCHARGE CHARACTERISTICS OF ELECTRIC GYMNOTID FISHES IN THE RIO NEGRO, BRAZIL

A. B. STEINBACH

Department of Physiology, University of California, Berkeley, California 94720

Fishes of widely differing evolutionary backgrounds possess organs specifically adapted to produce an external electric field. In most, the field produced by the electric organ is too weak to be of use as a weapon, hence the name "weakly electric fish," to distinguish them from the strongly electric freshwater electric eel (*Electrophorus*) and electric catfish (*Malapterurus*) or the marine *Torpedo* and Stargazers (Family Uranoscopidae) (Lissmann, 1951; Coates, 1947, 1954).

Lissmann and others have shown by behavioral tests that *weakly electric fishes* can detect changes in the conductivity of their surroundings (Lissmann, 1958; Lissmann and Machin, 1958; Machin and Lissmann, 1960; Wantanabe and Takeda, 1963). This is accomplished by using the electric organ as the energy source, and specialized cutaneous receptors as the receivers for a system of active electrolocation, although the actual mechanisms involved are different than first suggested (Machin and Lissmann, 1960; Bennett, 1967).

Both the structure and the physiology of the electric organ and the receptors have been studied in several *weakly electric fishes* (reviewed by Bennett, 1961, 1967, 1968). Yet despite experimental evidence showing that *weakly electric fishes* are capable of utilizing their unique sensory system, little research has been performed to determine the extent to which their usual behavior depends upon use of this system (Lissmann, 1961a, b). This article reports studies of weakly electric gymnotids.

Fishes were captured and studied in aquaria on board the R/V Alpha Helix. The movements of fishes in the river were followed by recording their electric discharges.

Study Area

The R/V Alpha Helix moored at 01°24 S, 61°27 W, downstream of the confluence of the Rio Branco and the Rio Negro rivers about 200 miles north of Manaus, Amazonas, Brazil. This point was about 160 m from an island near the right bank of the river. Average river width exceeded 1 km, and the average rate of flow at the ship was 0.59 knots (measured on May 5–7, 1967). Surface water temperature was about 28°C. Water near the right bank, of Rio Negro origin, was free of sediment but the color of weak tea; it will be called "black water" following common usage. Near the left bank, sediment filled water from the Rio Branco predominated; this will be called "white water." Fine sand and mud overlain with sunken leaves and branches, and occasional clusters of boulders, comprised the river bottom. An area near the right bank was studied most intensively. Varying sized spotches in Figure 1 appearing to be bushes growing along the right bank of the river were in fact large and small trees standing in water 6 m deep. River depth near the ship was determined with a portable Bendix echo sounder. By local report, the river undergoes a depth change of about 6 m annually, and much of the study area was usually above water during the dry season. During the study, 4 April to 15 May 1967, the river was approaching flood stage, and the forest surrounding the base camp on the island was covered with water.

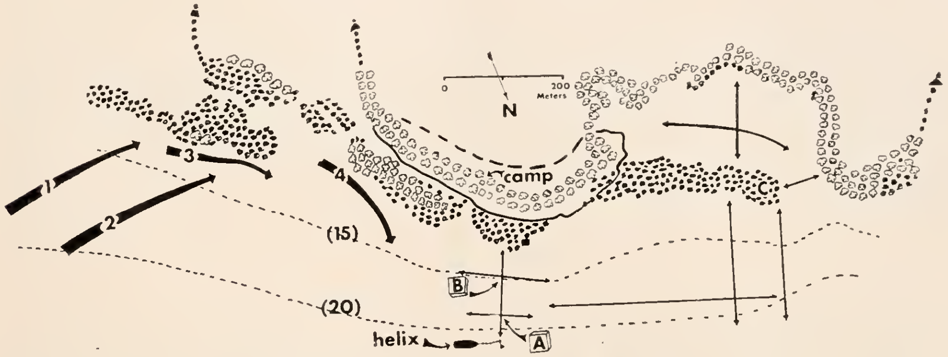


FIGURE I. Sketch map of the study area; "helix" denotes the R/V ALPHA HELIX anchored off an island on the right bank of the Rio Negro. The large and small splotches denote large and small submerged trees. Dry areas are shown by solid and dashed lines around the camp area. Heavily dashed arrows indicate continuations of the submerged trees and the island proper. The lightly dotted lines indicate approximate depth contours in meters. The large solid arrows (1–4) denote trawling locations. The thin lines mark locations where transects of activity were made. The boxed letters A and B show the location of marking buoys. Magnetic north and a scale of distance are noted.

METHODS

Electrical activity from fishes was detected by recording between two carbon electrodes fixed 0.4 m apart attached to a 26 m coaxial cable marked in 1 m gradations. Signals were amplified by a miniature transistor pre-amplifier and earphone (called "fish detector" henceforth) or recorded on magnetic tape with a Sony portable A.M. recorder. To obtain transects of activity *versus* position, these electrodes were dragged slowly behind a canoe over bottom transects. Tape recordings made during these transects were later integrated electronically (1 to 5 sec time constant). The integrated record was plotted out on a XY plotter as a function of time; by paddling at a constant speed over a known distance this procedure provided a record of activity *versus* position. This method weighted sinusoidal signals preferentially to pulsed signals, and thus could not be used to make quantitative comparisons between different species of fish. Identifications of pulse shape and frequency were made from original tape records with a Tektronix 502A oscilloscope.

Activities at two fixed locations were sampled at various intervals for several days and converted to numerical levels of integrated activity, for quantitative comparison. Other measurements of activity, of a less quantitative nature, were made by noting the amplitude of the signal heard with the fish detector. In controlled tests, fishes could be detected within a radius of about 2.5 m. Because signal strength varied with species, distance, and position of the emitting fish, no attempt was made to determine positions of individuals within this sampling radius. One or two fish could be distinguished from many individuals.

Gymnotids were found only under thick cover (shallow water) or at some depth, and were never seen underwater. They were collected with a 4 m otter trawl towed along the bottom, behind a power boat at a speed of about 0.5 knots. Trawls were made at various hours of the day and night, usually along the lines indicated by the heavy black arrows (labeled 1 to 4) in Figure 1. Fishes were observed in aquaria on board ship. Using an electronic counter, the exact frequency of discharge was determined for several individuals of each major species. Rough "maps" of the electric field produced by each specimen were made with a monopolar electrode, and the types and locations of electric organs were determined by dissection of fresh and formalin fixed specimens.

Observations

Collection and examinations of specimens

Several trawls were made along each of the tracks indicated (Fig. 1). The total catch composition, by species, is given in Table I, column 2, and was proportionally the same in all trawls. Pending a revision of the gymnotids, species designations follow Ellis's key (1913; see also Eigenmann, 1922; Schultz, 1949). During daylight hours (roughly 0600 to 1800 hrs) many fishes were caught trawling along tracks 1 and 2 (in deep water) and less than 5 per trawl along tracks 3 and 4 (near the shore). At night less than 6 fishes were trawled along tracks 1 and 2, while about 30 per trawl were caught along tracks 3 and 4.

Fish of a given species always showed the same waveform of discharge. Fish in group 2 (Table I, column 7) could change their frequency (but not waveform) of discharge when disturbed (Lissmann and Schwassmann, 1965; Mandriota, Thompson and Bennett, 1965; Wantanabe and Takeda, 1963); this was observed in aquaria and under natural conditions. With one exception, all other fish discharged at a constant frequency (Lissmann, 1958; 1961b). Individual black *Sternarchus* specimens were capable of abrupt transient increases in frequency, producing an audible chirp in the fish detector (Yoshika Oniki, Instituto Pesquesas Escola Agronomica Nacional, I. P. E. A. N., personal communication; Bullock, 1970).

Species could not be identified reliably by electrical criteria. Considerable overlap in ranges of discharge frequencies was observed (Table I, column 5), and waveforms of discharge were the same for 4 species (Group 1, Table I). For the 13 species examined, electrical criteria alone suggested 6 "groups" of fishes (Table I, column 7, Fig. 2). In all cases increase in temperature increased discharge frequency (Enger and Szabo, 1968). The range of body length for each species is noted in Table I. There was no correlation between body length and frequency of discharge.

TABLE I

Numbers of Gymnotids of various species and their size, and frequency of discharge. The fishes are also grouped according to frequency range and waveform of discharge

Species	Fish		Discharge frequency		Body length range (cm)	Group*	Discharge frequency range	Per cent† tot. sig.
	Caught	Studied	Mean	Range				
Sternarchella sp.	410	26	1200	932–1384	8–15	1	900–1300	40
Adontosternarchus sp. (var.)	372	21	1036	889–1241	6–10			
Sternarchus sp. (black)	58	12	1134	964–1466	13–16			
Adontosternarchus sp. (black)	20	8	1184	1053–1330	6–10			
Steatogenys sp.	219	15	55	40–70	7–15	2	35–80	35
Gymnorhamphicthys sp.	2	2	40	18–55	10			
Rhamphichthys sp.	2	1	40	20–55	10			
Eigenmannia virescens	19	5	320	300–360	10–15	3	300–800	10
Eigenmannia macrops	13	8	750	725–802	8–12			
Sternarchorhynchus sp.	17	8	1268	1020–1702	10–20	4	1100–1700	5
Sternarchorhamphus sp.	17	8	920	740–973	13–35	5	800–1000	5
Sternopygus-like (rough)	7	2		104–165	15	6	100–200	5
Sternopygus-like (smooth)	2	2		100–150	10			

* Number designations of groups are arbitrary. Fish were grouped by analysis of both frequency and waveform of discharge.

† Figures are estimated percentages of the electrical signal detected in the river that could be clearly identified as originating from a given group of fishes. The frequency range of discharge given for each group is based on analysis of tape recordings taken from the river.

There were considerable differences in behavior within the group of fishes having virtually identical discharge characteristics (group 1). For example, although the fish called black *Sternarchus* sp. aggregated in aquaria, individuals showed extreme intra-specific interaction, probably aggressive, manifested by approaches, bumping attacks and chasing (Yoshika Oniki, personal communication). In aquaria these fish showed some interest in black *Adontosternarchus* sp., a smaller fish with a very similar waveform and frequency range of discharge, but

TABLE II

Electrical activity recorded with the fish detector at various locations underwater within three miles of the ship anchorage. Intensity of activity is denoted by one or more plus signs (+) *and no activity by a zero* (0), *while no or insufficient data are indicated with a bar* (–). *Night time activity along the left bank was not studied.*

Location	Depth (m)	Right bank		Left bank
		Day	Night	Day
Small streams and inlets	0–4	+	–	+
Flooded trees	2–6	0	0	+
Backwater channels	6–20	0	0	++
Perimeter of trees	6	0	+++	0
River near trees	6–10	0	+	–
River main channel	10–20	++	0	++

did not engage in the displays observed between two black *Sternarchus* sp. The black *Adontosternarchus* sp. were not aggressive, and were generally less active than the black *Sternarchus* sp.

In aquaria, all of the fishes tended to gather together in species specific aggregations to some extent. *Steatogenys* sp. formed particularly close aggregations, with no apparent intra-specific aggressiveness, and showed a clear day-night activity cycle, preferring to hide under available cover during the day. They comprised the major part of group 2 (Table I).

TABLE III

Comparisons of intensities of electrical activity obtained by sampling in four areas during the day and night (Fig. 1). Each number is the average intensity recorded during a one minute period with recording electrodes on the bottom. The arbitrary scale 0 to 5 was derived by calibrating the gain adjustment on the fish detector and is not the same as the scale shown in Figure 5.

Depth and location in Figure 1.	Activity (scale 0 to 5) during hours 0900–1700	Activity (scale 0 to 5) during hours 1700–0600
16 meters	4, 3, 1, 1, 3, 2	0, 1, 1, 1, 1
Right hand side	3, 1, 1, 2, 1	2, 0, 0, 0, 0
16 meters	4, 3, 1, 1, 3, 2	0, 1, 1, 1, 1
Right hand side	3, 1, 1, 2, 1	2, 0, 0, 0, 0
12–18 meters	5, 5, 2, 3, 5	0, 0, 0, 1
Right middle		1, 2, 0, 3, 0
18 meters	2, 3, 3, 2, 2	0, 0, 0, 1, 1, 0
Left hand side	1, 1, 3, 1	0, 2, 1, 1, 1, 1
6 meters	0, 0, 0, 0, 0, 0	5, 5, 4, 3, 5
Along trees		4, 5, 3, 3, 5

Each trawl took about 10 minutes, and although little debris was picked up, the net obviously damaged many specimens. The most numerous species (variegated *Adontosternarchus* sp. and *Sternarchella* sp.) had the highest mortality rate; less than 30% of each catch survived for more than 2 hours after capture. The stomach contents of all species were rather similar, although *Sternarchella* sp. usually had a quantity of fine sand in the stomach that was not found in the other species. Insect larvae, isopods, filamentous algae, and portions of unidentifiable exoskeletons were found in all stomachs. Stomach contents were larger and better preserved in fish collected in the early morning, which would support a hypothesis of nocturnal feeding.

Of the 13 species collected and examined by dissection (species represented by a single specimen are not listed) those in groups 2, 3 and 6 (Table I) had electric organs of muscular origin (Szabo, 1966; and review by Bennett, 1961), while those in the other groups had organs derived from nerves (Oliveira-Castro, 1955; Bennett, 1966). Thus as a rule low frequency fishes have organs linked to spinal neurones by a chemically transmitting synapse, while the organs of high frequency fishes are themselves modified spinal neurones. In all cases the main electric organ or organs were located primarily in the posterior part of the body, sometimes extending forward to a point anterior to the visceral cavity (Bennett, 1961, 1968). Fish in group 2 had auxiliary organs also of muscular origin in the head (Bennett, 1962).

Localization of electrical activity

Table II summarizes the intensities of electrical activity recorded with fish detector at various locations underwater in or near the main river. No activity was ever detected within the flooded (igapo) forest along the right bank. Low frequency activity was detected within the igapo forest along the left bank. Only low frequency activity was detected in small streams and inlets and single specimens of *Hypopomus* sp. and *Steatogenys* sp. were captured there. Much of the daytime activity detected in deep water, and also along the submerged lines of trees at night, was high frequency (Table I, column 8, 9). On a basis of frequencies and waveforms of activity, 6 groups of fishes were identifiable, corresponding to the six groups of electrically distinct fishes studied in aquaria (Table I, column 7).

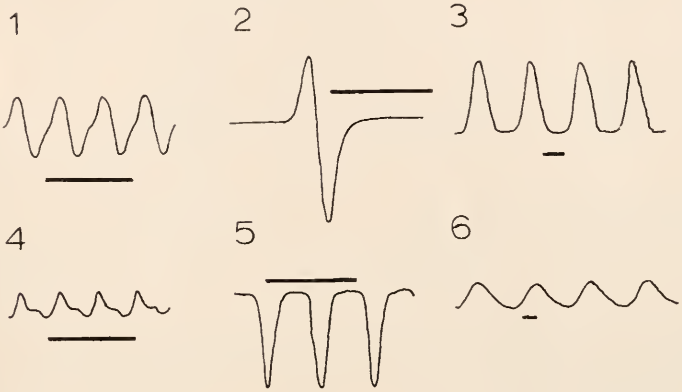


FIGURE 2. Characteristic waveforms of discharge for groups of fishes (Table I). Recordings were made with a monopolar electrode placed near the head; a head positive deflection is upwards. A time calibration bar indicates 2 msec (1 msec for waveform 2); the maximum signal detected was about 50 mV.

Table II indicates that locations where activity was highest during the day became silent at night, and vice-versa. Activity during the day in deep water appeared to be concentrated in patches, although always at or near the bottom. At night, activity was spread continuously along the tree line, from about 1 m to the bottom (Table III).

Particularly intense patches of daytime activity were found just inshore from the ship anchorage (Fig. 3). A rocky area extending underwater from the shore probably provided secure resting places for the fishes. Transects of activity were made across this area at various times of day and night on a total of five days. Typical results for a transect made on four occasions from near the ship mooring towards the shore are depicted in Figure 4. The second, third, and fourth traces, taken during daylight hours, indicated two roughly constant patches of

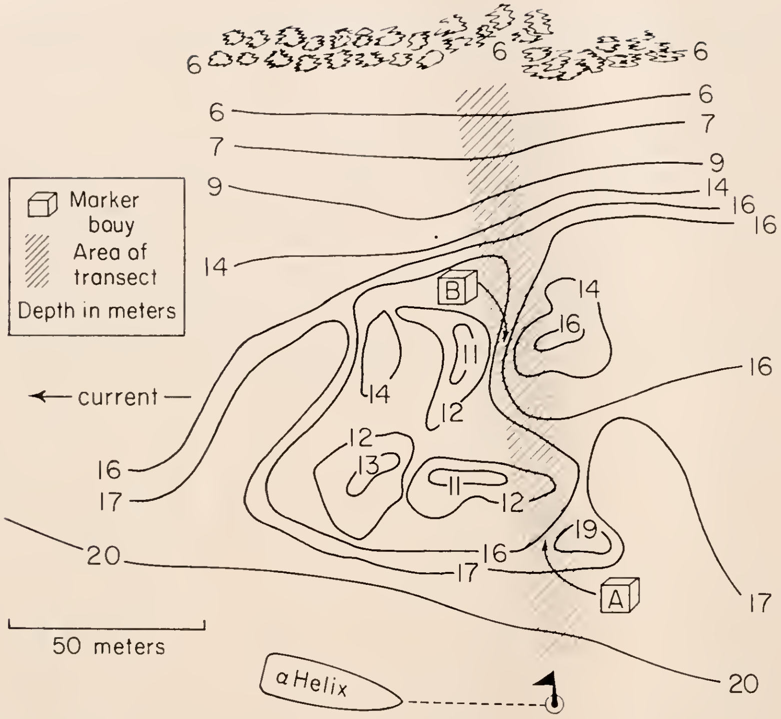


Figure 3. Depth contours of the river bed inshore from the ship anchorage. The steep drop-off from 6 to about 15 meters and the drop at 20 meters are typical of the river as a whole; the irregularities shown indicate the rocky submerged point extending from the shore (the line of submerged trees is indicated by splotches). The path taken during the transects illustrated in Figure 4 is shaded.

activity. The type of activity is indicated by a number (referring to the groups in Table I) below each trace. Artifacts (subscripted "A") resulted when the electrodes truck rocks. The lowest trace (heavy line) indicates depth along the transect. Practically all activity along the entire transect vanished during the night (compare the upper trace with others; the recording gain was about 10 times that used in the third trace, and 5 times that in the second and fourth traces). Thirteen other transects (not illustrated) along the lines shown in Figure 1 indicated that activity was restricted to patches, and was not distributed along the river bed at a constant depth or distance from shore.

To determine the stability of the deep water activity, two anchored buoys (marked A and B in Figs. 1, 3 and 4) were placed over areas of high daytime activity, and integrated bottom activities below these buoys were determined at intervals (Fig. 5). During the day, activity at buoy A was emitted exclusively by group 2 fishes, while that at buoy B was a mixture of groups 1 and 3. On successive days, activity at buoy A was constant to within at least ±10%, while that at buoy B was slightly more variable, ±25%. Each aggregation was found to be about 3 m in radius. Between 1600 and 1800 hrs, as the sun set, activity began

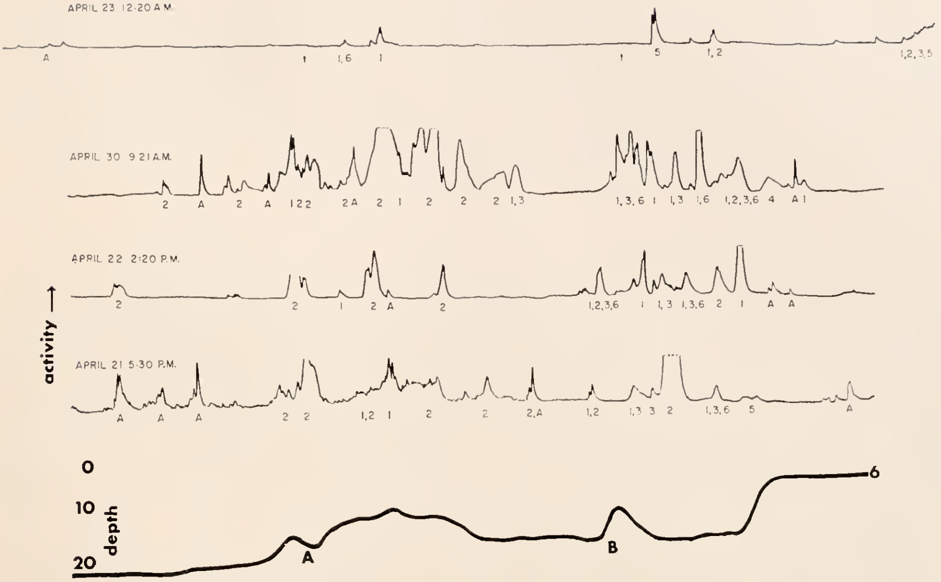


FIGURE 4. Transects of electrical activity versus position (date and time as noted), taken along the path indicated in Figure 3 and plotted by the method described in the text. Net gain of the electronic apparatus used was 5 times higher in the first trace than in the second and the fourth, and 10 times higher than in the third. The numbers under activity peaks refer to identified groups of fish (Table I). Artifacts are indicated by the letter A. The heavy line trace shows in depth in meters. In each case the distance covered was about 140 m, but during the uppermost trace paddling speed was slower, and thus the trace is somewhat longer. It may be seen that the major peaks of activity appear in the same places at all times, and that at night virtually all activity is lost.

to decrease at buoy A. At buoy B, activity of group 1 and 3 lessened, but was replaced, towards 1900 hrs, by activity of group 2. After 2000 hr activity declined at both buoys, and by 2100 hr what little remained consisted of a mixture of all groups (mostly single specimens) at buoy A and group 1 and 2 at buoy B.

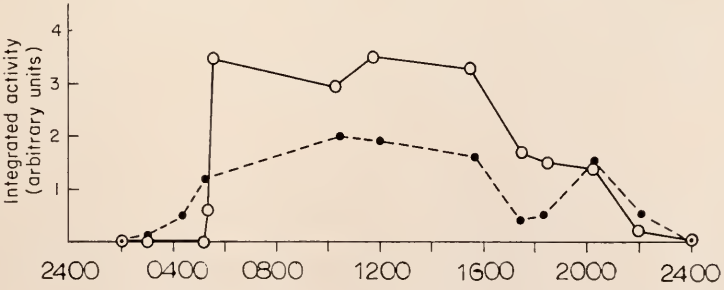


FIGURE 5. Plot of integrated activity in arbitrary units versus time in hours for one twenty four hour day. Each point indicates one measurement; the solid lines and open circles O——O record activity at buoy A and the dashed lines and filled circles (●- - -●) that at buoy B (Fig. 3).

By 2400 hrs there was essentially no activity at either buoy. Single fish were detected at both buoys on some occasions. Near the submerged trees, activity reached a maximum at about 2300 hr and remained at a high level (all groups intermixing) until about 0430 hr. At about 0500 hr a mixture of all groups of activity was usually detected at buoy B, but nothing at buoy A. Within about 20 minutes the activity at buoy A had reached daytime level.

Discussion

More than 1000 gymnotids were trawl collected, representing at least 13 species. Because of overlaps in frequencies of electrical discharge and also similarities in waveform of discharges, only 6 groups were distinct using purely electrical criteria. The same 6 electrical groups were identified from analysis of activity recordings made under natural conditions. It seems probably that the species studied in aquaria fairly represent the species studied under natural conditions. The percentage of total activity attributable to each group in recordings made under natural conditions was about the same as the percentage composition by a group of the fishes collected in trawls.

Electrical activity was intense in deep water and absent along the shore during the day. Fishes were plentiful in deep water and scarce in shallow water during the day as indicated by numbers caught in trawls. With regard to both electrical recordings and trawling this situation reversed at night. These observations support the belief that electrical methods were of value in following the diurnal movements of the fishes.

The fishes studied resemble those previously reported by others with regard to body form, origin and location of electric organs, and waveform and frequency of electrical discharges (see Lissmann, 1958, 1961b; Bennett, 1961, 1968). It is interesting that fishes of different species whose electrical characteristics appear to be identical can quite clearly recognize members of their own species. Presumably not only the electrical discharges but the changes in discharge waveform as a fish moves are important in species recognition, just as movements (displays) are important to visually oriented animals.

Fish belonging to group 2 (probably *Steatogenys* sp.), aggregating at buoy A, made diurnal migrations over a distance of at least 100 m, and from a depth of about 16 m to the surface along the tree line. There was no reason to believe that the fish left the bottom in midstream. They might have navigated either by smell or by feel. However, electrical recordings from fixed locations indicated that the same or an equivalent number of fish returned to the same spot day after day within a very short time period each morning. Since the fishes dispersed at night along the trees, they apparently have an ability to reaggregate as well as to navigate over relatively long distances. Electroreception is not the only sense that could produce this ability, but it would certainly be of use under the conditions in which the fishes live. The constancy of activity during the day near buoy A suggests that the fish remained quiescent; this was supported by aquarium observations of *Steatogenys* sp.

Both high and low frequency fish were found throughout the study area near the main course of the river, and apparently shared the same feeding ground at night. The high frequency fishes were more abundant in the river (Table I), and were not found in the shallow waters along either bank. No gymnotids were found in the semistagnant black water in swamps along the right bank, perhaps because there was little food available in this water. The fish studied were smaller than those of the same or similar species studied elsewhere (Ellis, 1913), again perhaps because of the low nutrient content of black water.

Lissmann (1961b) found using electrical recording in the field that gymnotids near Belem, Brazil living in relative shallow clear water dispersed from daytime hiding places among rocks, weeds or trees and became active at night. This behavior would effectively avoid visually oriented predators (Lissmann, 1961b). In the present study, potential predators were not captured in trawls. The gymnotids captured had small mouths and teeth. Possibly predation becomes significant only during low water when, by local account, electric eels, piranhas and many other potential predators frequented the study area. Captive electric eels avidly pursued and consumed weakly electric fishes and appeared to be homing in on the electric signal during pursuit as evidenced by increased average time required to catch spinalized fishes lacking electrical discharges. Electric eels respond behaviorally to electrical signals, and possess electroreceptors similar to those of other gymnotids (Hagiwara, Szabo and Enger, 1965). However, the failure to catch predators in the trawl may simply reflect inadequate methods.

The extreme annual changes in water level may partially explain the diurnal movements of the gymnotids. During the dry season, most of the vegetation shown in Figure 1 would be on dry land. The fishes may choose to maintain resting places in water that does not disappear seasonally, rather than simply moving with the changing waterline.

The movements of the fishes studied, and their species specific daytime aggregating tendencies do not, of course, prove that the fishes use electroreception for either navigation or species recognition. However, because of the opaque, deep water in which the fishes live, it is obvious that their known electrosensory abilities would be of considerable advantage. The extent to which electroreception actually determines behavior is a question that remains for more controlled experimentation to answer.

I am especially indebted to Captain Faughn and the crew of the Alpha Helix for their help, to members of the scientific party for their suggestions and criticism, and to the National Science Foundation for supporting the 1967 Ouro Verde Amazon expedition. I also wish to thank The Rockefeller University, at which I was a student, for a travel grant and for the loan of equipment.

Summary

Gymnotids of 13 species were trawled in water 10–20 m deep; all emitted weak electrical discharges. Each fish had a typical frequency of discharge; each species had a typical range of frequency but there were overlaps between species. Waveforms of discharge were similar in several species. Electric organs were derived from muscle tissue in most low frequency species and from nerve tissue in high frequency species. Diurnal movements of fishes were followed electronically.

Fishes formed aggregations in 15–20 m deep water about 100 meters from shore during the day and dispersed along the shore at night. Individual daytime aggregations were re-established each morning.

LITERATURE CITED

Bennett, M. V. L., 1961. Modes of operation of electric organs. *Ann. New York Acad. Sci.*, **94**: 458–509.

Bennett, M. V. L., 1962. Electric organs of the knifefish *Steatogenys*. *J. Gen Phsiol.*, **45**: 590A.

Bennett, M. V. L., 1966. An electric organ of neural origin. *Fed. Proc.*, **25**: 569.

Bennett, M. V. L., 1967. Mechanisms of electroreception, pp. 313–393. *In:* P. Cahn, Ed., *Lateral Line Detectors.* University of Chicago Press, Chicago, Illinois.

Bennett, M. V. L., 1968. Neural control of electric organs, pp. 147–169, *In:* D. Ingle, Ed., *The Central Nervous System and Fish Behavior.* University of Chicago, Chicago, Illinois.

Bullock, T. H., 1970. Species differences in effect of electro-receptor input on electric organ pacemakers and other aspects of behavior in electric fish. *Brain, Behavior and Evolution,* **2**: 85–118.

Coates, C. W., 1947. The kick of an electric eel. *Atl. Mon.*, **180**: 75–79.

Coates, C. W., 1954. What we are learning about electric fishes. *Anim. Kingdom,* **57**: 182–186.

Eigenmann, C. W., 1922. The fresh water fishes of north-western south America. *Memoirs of the Carnegie Museum.* **9**: 1–346.

Ellis, M. M., 1913. The gymnotid eels of tropical America. *Memoirs of the Carnegie Museum.* **6**: 109–204.

Enger, P. S., and T. Szabo, 1968. Effect of temperature on the discharge rates of the electric organs of some gymnotids. *Comp. Biochem. Physiol.*, **27**: 625–627.

Hagiwara, S., T. Szabo and P. S. Enger, 1965. Physiological properties of electroreceptors in the electric eel, *Electrophorus electricus*. *J. Neurophysiol.*, **28** : 775–783.

Lismann, H. W., 1951. Continuous signals from the tail of a fish, *Gymnarchus niloticus* Cuv. *Nature,* **167** : 201.

Lissmann, H. W., 1958. On the function and evolution of electric organs in fish. *J. Exp. Biol.,* **35**: 156–191.

Lissmann, H. W., 1961a. Zoology, Locomotory adaptations and the problems of electric fish, pp. 103–124. *In:* J. A. Ramsay, Ed., *The Cell and the Organism.* Cambridge University Press, Cambridge, Massachusetts.

Lissmann, H. W., 1961b. Ecological studies on gymnotids, pp. 215–223. *In:* C. Chagas and A. Paes de Carvalho, Eds., *Bioelectrogenesis.* Elsevier, Amsterdam.

Lissmann, H. W., and K. E. Machin, 1958. The mechanism of object location in *Gymnarchus niloticus* and similar fish. *J. Exp. Biol.*, **35**: 451–486.

Lissmann, H. W., and H. O. Schwassmann, 1965. Activity rhythms of an electric fish *Gymnorhamphichthys hypostomus* Ellis. *Z. Vergl. Physiol.*, **51**: 153–171.

Machin, K. E., and H. W. Lissmann, 1960. The mode of operation of the electric receptors in *Gymnarchus niloticus*. *J. Exp. Biol.,* **37**: 801–811.

Mandriota, F. M., R. L. Thompson and M. V. L. Bennett, 1965. Classical conditioning of electric organ discharge rate in Mormyrids. *Science,* **150**: 1740–1742.

Oliveira-Castro, G. de, 1955. Differentiated nervous fibers that constitute the electric organ of *Sternarchus albifrons* L. *Anais da Academia brasileira de ciencias,* **27**: 557.

Schultz, L. P., 1949. A further contribution to the ichthyology of Venezuela. *Proc. U. S. Nat. Mus.,* **99**: 1–211.

Szabo, T., 1966. The origin of electric organs of *Electrophorus electricus*. *Anat. Rec.,* **155**: 103–110.

Wantanabe, A., and K. Takeda, 1963. The change of discharge frequency by a-c stimulus in a weak electric fish. *J. Exp. Biol.,* **40**: 57–66.

Reference: *Biol. Bull.*, **138**: 211–218. (April, 1970)

OBSERVATIONS ON THE FUNCTION OF THE FRONTOLATERAL HORNS AND HORN GLANDS OF BARNACLE NAUPLII (CIRRIPEDIA)

PETER B. TAYLOR [1]

Department of Oceanography, University of Washington, Seattle, Washington 98105

Frontolateral horns and associated glands are prominent, characteristic features of the nauplii of most Cirripedia. Frontolateral horns are possessed by the orders Thoracica (including the Balanomorpha, Verrucomorpha, and Lepadomorpha), the Acrothoracica, and the Rhizocephala, but not by the Ascothoracida. It is by the frontolateral horns that the nauplii of true cirripeds can be distinguished from those of other Crustacea. They must serve some function, and this paper is concerned with a hypothesis that the frontolateral horns and horn glands constitute venomous organs that serve barnacle nauplii as a defense mechanism against predation.

Balanus perforatus nauplii (Balanomorpha), as described by Groom (1894), have frontolateral horns that are typical of barnacle nauplii in general (Fig. 1). The frontolateral horns and horn glands of the Acrothoracica and the Rhizocephala are essentially similar to those of the Thoracica (Krüger, 1940). The horns are tubular structures projecting from the dorsal shield. Their tips are perforate. Within each horn is a spindle-shaped organ consisting of two closely applied gland cells. The "frontolateral horn glands" extend from a point near the middle of the dorsal shield, above the gut, into the lumen of each horn. Transparent globules of secretion may fill the lumen. Groom (1894, page 193) described the globules, which he said are ". . . provided with a resistant pellicle, and which, though closely pressed against one another into polyhedral bodies, . . . do not fuse; they are not dissolved by water, alcohol, weak acids or alkalies; they show no acid or alkaline reaction and take up no colouring matter." He also speculated (page 193) that the membranes of the glands and of the globules are chitinous, but pointed out that "the chitin . . . must be of a delicate nature, for while resembling the cuticular covering of the whole body in being soluble in warm acid (HNO_3), it dissolves in hot caustic potash, which ordinary cuticle resists."

To my knowledge, Groom's are the most detailed descriptions of the horns, horn glands, and secretion that exist for any cirriped nauplii. Kaufmann (1965), however, presented photomicrographs of the horns and horn glands of *Scalpellum scalpellum*. Maximum development of the horns is attained among the Lepadomorpha, for example in the genus *Lepas* (Groom, 1894, Figure 156).

In the thoracican barnacles, the horns and horn glands are initially formed during later embryonic stages (Groom, 1894; Krüger, 1940). Stage I nauplii have

---

[1] This research was supported by a National Science Foundation Postdoctoral Fellowship to the author and by grant GB-3551 from the National Science Foundation to the University of Washington. Contribution No. 519 from the Department of Oceanography, University of Washington, Seattle, Washington, and Contribution No. 157 from the Systematics-Ecology Program, Marine Biological Laboratory, Woods Hole, Massachusetts.

the horns directed posteriorly along the lateral margins of the dorsal shield and the tips are closed. Following the first molt and for the five subsequent naupliar stages, the horns are directed anterolaterally and somewhat ventrally. The horn tips are perforate and complex, consisting of terminal stylets and spine processes. The horns appear to become vestigial during metamorphosis of the metanauplius (Stage VI) to the cyprid larval stage. In the cyprids of *Scalpellum scalpellum* they are reduced to closed, short, terminally widened, funnel-shaped stumps into which the glands open (Kaufmann, 1965).

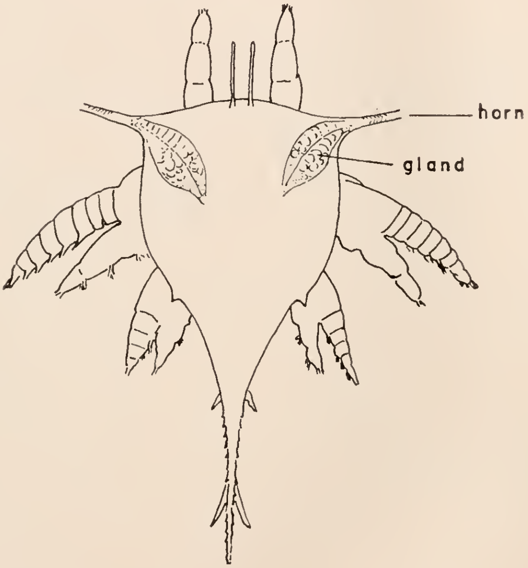


FIGURE 1. The stage II nauplius of *Balanus perforatus* (after Groom, 1894, Fig. 141); setation omitted; Horn = frontolateral horn, Gland = frontolateral horn gland.

Speculations on the function of the frontolateral horns and horn glands were advanced during the nineteenth century. Darwin (1851), regarding the frontal filaments as a pair of appendages, professed to see within the horns the developing second pair (second antennae). Dohrn (1870) considered the horns and their glands as possible precursors of the adhering organs of the cyprid. We know now that the frontal filaments and the horns are not true appendages and that the glands of the true first antennae are the adhering organs.

Groom (1894, page 193) pointed out that in the Thoracica the period of secretion of the horn glands coincides with the free life of the larvae, during part of which they feed. He agreed with Claus and with Hoek (cited by Groom, page 193) that the sharp points and spines at the ends of the horns indicate that they may be piercing organs provided with poison glands. He further observed ". . . that the area covered by the horns is that included by the sweep of the appendages, and that any organism paralyzed by the secretion would tend to be swept towards the region of the mouth." Groom thus proposed a feeding function.

Recently, Drs. W. A. Newman and S. A. Wainwright reported observations (personal communications) from which they inferred that the frontolateral horns and horn glands might be venomous or noxious organs for defense against predation. Newman observed an increased mortality of shrimp (*Palaemon*) larvae in culture following the introduction of (*Balanus*) nauplii; Wainwright noted the vigorous withdrawal of coral (*Pocillopora*) tentacles from contact with barnacle (*Balanus*) nauplii in laboratory observations. My observations, reported here test the hypothesis of the existence of a venomous defense mechanism. The observations were made at the Marine Biological Laboratory, Woods Hole, Massachusetts, and at the Friday Harbor Laboratories (University of Washington), Friday Harbor, Washington.

MATERIALS AND METHODS

Barnacle nauplii reared in the laboratory were used for most trials. Rearing methods were based on those of Moyse (1960). Cultures were initiated from embryos removed from the mantle cavities of adult barnacles. Usually 200 to 400 nauplii were reared in each 1-liter glass Berzelius beaker containing paper-filtered or membrane-filtered (Millipore HA 45 μ) seawater. Portions of unialgal cultures of *Skeletonema costatum, Cyclotella nana, Cyclotella cryptica,* and *Thalassiosira fluviatilis* were added for food. Continuous light was provided to maintain algal growth in the rearing vessels. At Woods Hole, the vessels were maintained near 12° C in a constant-temperature room; at Friday Harbor they were kept on a running seawater table at temperatures between 11 and 13° C, near local surface water temperature. The algal cells were resuspended daily by stirring and were replenished if depleted. The *Cyclotella* spp. were the most satisfactory of the algae used because they remained in suspension and grew rapidly at rearing-vessel temperatures. Nauplii were transferred to clean vessels and seawater containing fresh algal culture at least weekly. In this way embryos of *Balanus balanoides, B. balanus, B. improvisus,* and *B. glandula* were reared to cyprids. The success of the culturing methods was further evidenced by the fact that cyprids settled as spat in rearing vessels when subsequently left undisturbed. Attempts to rear *Pollicipes polymerus* by these methods failed beyond Stage II nauplii.

"Wild" barnacle nauplii were isolated from plankton net collections (153-μ-mesh, 30-cm-diameter net) from near the water surface. They were not identified beyond genus.

Brine shrimp (*Artemia salina*) nauplii were hatched and reared in fingerbowls containing filtered seawater plus portions of unialgal cultures, kept under lights at ordinary room temperatures.

Potential predatory species were isolated from plankton-net collections, or were dip-netted by day (or at night under a submerged light); benthic forms were collected intertidally. These animals were generally held in 16-oz (473-ml) low-form plastic containers or glass jars on a seawater table to maintain lower temperatures. *Artemia* nauplii were supplied as food. Planktonic crustaceans seemed to survive best in vessels made dark by enclosure in black plastic.

OBSERVATIONAL METHODS

The direct approach was to observe the reactions of potential predators to barnacle nauplii. Barnacle nauplii were pipetted into a vessel usually containing a potential predator. Behavior was observed directly or with the aid of a dissection microscope. For comparison, the reactions of predators to *Artemia* nauplii were observed. These reactions also tested the predators' predisposition to feed before the trials. Stage I barnacle nauplii, presumably "unarmed" because the frontolateral horns were not yet erect or perforate, also served as controls. The warming of animals above maintenance temperatures was minimized by working in a cold room or by observing for only short periods and using large water volumes.

Survival trials were also conducted to avoid the conditions of continuous bright light and handling, which might interfere with the normal behavior of the barnacle nauplii or the potential predators. Usually 10 barnacle nauplii were introduced into a 16-oz (473-ml) low-form wide-mouth glass jar containing a single predator. Predator mortality, the consumption of barnacle nauplii, or other effects were then determined through periodic censuses. *Artemia* nauplii and Stage I barnacle nauplii were used as controls in separate vessels, and were cointroduced with later-stage barnacle nauplii. Still other controls confirmed survival of the nauplii in the vessels without predators. Between censuses, all vessels were kept on a running seawater table for temperature control, and all except those containing medusae were darkened by black plastic enclosures. Censuses of the number and condition of the animals were made under oblique or side lighting against a black background. Predation was best demonstrated by the presence of prey remains in the guts of the predators, or in their fecal material examined under a dissection microscope, because counts of surviving nauplii were difficult to make and did not prove the fate of missing individuals.

Results

Direct observations on the behavior of certain potential predators toward barnacle nauplii are summarized in Table I. Other animals (chaetognaths, euphausids, predatory copepods, decapod zoeae, medusae, and fish postlarvae) were tried but failed to feed under the viewing conditions. Several representatives of the Cnidaria and bony fishes captured and consumed barnacle nauplii, and did so without ill effects. On the other hand, for two hydromedusae, *Nemopsis bacheii* and *Aequorea aequorea,* the results shown in Table I are perhaps consistent with the defense hypothesis. Both species were indifferent to contact with the later stages of barnacle nauplii.

Results of the survival experiments are summarized in Table II. *Epilabidocera amphitrites* and *Neomysis rayii* consumed later-stage barnacle nauplii as readily as *Artemia* nauplii. Evidences of feeding by the copepod *E. amphitrites* were the folded but intact exoskeletons of barnacle nauplii appearing in fecal material deposited in the vessels. Nine such remains were found during the trials. *Artemia* nauplii exoskeletons were similarly found. I also noted (21 instances) the empty exoskeletons of barnacle nauplii in the vessels. Under the microscope these appeared to have been cut across the dorsal shield and some were partially crumpled. They were not exuviae from molting since they were never noted in controls or in trials with other predators. I conclude that the copepods probably were cutting the nauplii, extruding or sucking out the contents, and rejecting the

TABLE I

Observed reactions of potential predators to barnacle nauplii

Potential predators		Barnacle nauplii reactions*		
Species	No. of individuals	Source	Stage I	Other stages
		B. balanoides, Woods Hole		
Anthozoan				
Sagartia sp.	1	cultured		+
Hydroid colony				
Coryne sp.	1	wild		+
Hydromedusae				
Nemopsis bacheii	1	cultured		–
		B. glandula, Friday Harbor		
Sarsia tubulosa	1	cultured	+	
	6	cultured		+
	1	wild		+
Aequorea aequorea	1	cultured	+	
	1	cultured		–
Fish postlarvae				
Sebastodes sp.	1	cultured		+
	1	wild		+
Cottidae	1	cultured		+
	1	wild		+

* + = nauplii captured and ingested; – = no reaction, indifference to contact.

empty exoskeletons. Evidences of this behavior with *Artemia* nauplii were not observed. In neither type of prey was the exoskeleton fragmented, whether it was ingested or rejected. In contrast, fecal material from the mysid *Neomysis rayii* produced only fragments of exoskeletons from barnacle and *Artemia* nauplii. These observations indicate the need of caution in attempting to determine feeding habits of animals from their gut contents without knowing their behavior.

TABLE II

*Results of survival experiments**

Potential Predators	*Balanus*		*Pollicipes*	
	Stage I (cultured) +/–	Other stages (cultured) +/–	Other stages (wild) +/–	Other stages (cultured) +/–
Hydromedusae				
Aequorea aequorea	6/2	1/16		1/7
Phialidium gregarium	3/0	2/5		
Sarsia tubulosa	1/0	6/0	2/0	
Copepod				
Epilabidocera amphitrites		19/0	2/0	
Mysid				
Neomysis rayii		10/0	4/0	2/0

$$* \text{ Numerical data} = \frac{\text{number of trials, nauplii consumed } (+)}{\text{number of trials, nauplii not consumed } (-)}.$$

Among the hydromedusae the results of the survival experiments for *Aequorea aequorea* were consistent with the direct observations. *Aequorea* consumed Stage I and *Artemia* nauplii, but generally not later-stage barnacle nauplii. These data result from direct examination of the guts of living medusae, which are sufficiently transparent for the stages to be distinguished under low magnification.

Phialidium gregarium consumed barnacle nauplii of all stages but captured them less rapidly than Artemia nauplii, whether introduced together or separately. *Sarsia tubulosa* consumed all prey equally readily. On several occasions one or both frontolateral horns of an ingested barnacle nauplius were seen penetrating the wall of the manubrium without apparent harm.

For reasons mentioned later in the discussion of results, a special attempt was made at Friday Harbor in 1967 to view the reactions between *Balanus* nauplii and specimens of a ctenophore, *Pleurobrachia bachei.* The results were mixed. Poor condition of the *Pleurobrachia* specimens may have been a factor. They were collected during their reappearance following a dramatic decline in availability associated with exceptional warming and lowered salinity in the surface waters of Puget Sound and the Friday Harbor area in June 1967. *Balanus* nauplii likewise were not abundant in the zooplankton. Of the many *Pleurobrachia* specimens used, only a few indicated a readiness to feed by extending their tentacles in gently swirling water. Only three ingested food offered during the observations. One of these captured and ingested *Artemia* nauplii as fast as they were supplied, taking eight within 40 minutes. Of ten *Balanus glandula* nauplii (cultured Stages II and III) supplied, two adhered to tentacles, but they later swam free. Another *Pleurobrachia* specimen took three *Artemia* nauplii; two *Balanus* sp. "wild" nauplii adhered but were not ingested. A third specimen captured and ingested one *Artemia* nauplius and two *Balanus* sp. nauplii ("wild" Stages IV and VI). These and many more observations of contact showed that *Artemia* nauplii adhered more frequently to *Pleurobrachia* tentacles than did *Balanus* nauplii. Differences in mechanical stimuli may be a factor, although the nauplii were comparable in size and swimming rate. Copepods, larger and faster, were more consistently trapped in a tangle of tentacles.

Discussion

The results show that there are animals that can consume barnacle nauplii regardless of the frontolateral horns and horn glands. Indeed, *Balanus* nauplii have been listed among the stomach contents of postlarval fishes (Lebour, 1920; Sanders, 1952; Wailes, 1936) and the medusae *Phialidium hemisphericum* and *Aurelia aurita* (Lebour, 1922; 1923). Hardy and Bainbridge (1954) observed *Aurelia aurita* feeding on *Balanus* nauplii in the laboratory. These reports, however, do not disprove the defense hypothesis, because stage determinations were not mentioned; the nauplii might have been in "unarmed" Stage I. The present observations of the consumption of later-stage barnacle nauplii by a variety of predators and the passiveness of the nauplii to these predators would seem to rule out a generalized use of the horns as a venomous defense mechanism. Such use, however, under more specific circumstances or against a restricted class of predators, is not disproved in view of the results with hydromedusae.

The insolubility of the secretion in aqueous solvents, observed from living and freshly crushed specimens in this study and by Groom (1894), argues further against the secretion of a venom in the sense of a toxin to be introduced into the circulation of a victim.

That the frontolateral horns and horn glands are major features of barnacle nauplii emphasizes their importance. The glands secrete externally and are expected to be involved in behavior or excretion. The feeding function postulated by Groom (1894) is negated by the nonpredatory habits of the nauplii and by the fact that Rhizocephelan nauplii have well-developed horns and horn glands but do not feed. The nonaqueous nature of the secretion does not support involvement in social communication, as an attractant or a repellant, or in excretion. Other possible purposes for the secretion are in metamorphosis or cuticle formation, or in buoyancy adjustment.

The most attractive hypothesis suggested by the present observations is that the nauplii secrete through the horns a surface-active substance that prevents capture by animals that capture prey by use of adhesive organs. Cnidarians, including the hydromedusae, bear a variety of nematocysts, including some that are sticky rather than venomous (Russell, 1953). Dependence on this kind of nematocyst by *Aequorea* or *Phialidium* could explain their pattern of feeding on *Balanus* nauplii. Ctenophores depend on adhesive organs (colloblasts) for capturing prey. The observations on *Pleurobrachia bachei,* although inconclusive, did not support this hypothesis.

A surface-active agent might also prevent entrapment of the nauplii at the sea surface. *Balanus* nauplii are characteristically positively phototactic. In the present work they congregated near the air-water interface of culture vessels lighted from above but did not become trapped at the interface in the way copepods and amphipods often do. The contrast, of course, can be explained by the ability of the latter to penetrate the water surface by vigorous movements.

How barnacle nauplii use the frontolateral horns remains a mystery. A study of the chemical nature of the secretion, probably using histochemical techniques, seems to offer the best possibility for further clues of their function.

I am grateful to Dr. William A. Newman for bringing the problem to my attention and for reading the manuscript. Discussions with Drs. Dora P. Henry and Stephen A. Wainwright were helpful. I wish to thank Dr. Melbourne R. Carriker, Director, Systematics-Ecology Program, Marine Biological Laboratory, Woods Hole, Massachusetts, and Dr. Robert L. Fernald, Director, Friday Harbor Laboratories, University of Washington, for providing accommodations for this research. Miss Dora R. May and Mr. Peter J. Blanton provided technical assistance.

SUMMARY

1. Barnacle nauplii characteristically have frontolateral horns and horn glands, but the function of these is unknown. To examine the hypothesis that they are venomous organs that serve in defense against predation, observations were made on living specimens in the presence of potential predators.

2. Laboratory observations of two general types were made on *Balanus* spp. nauplii. One was to observe directly the behavioral interactions between the nauplii and potential predators. The other involved survival trials, in which barnacle nauplii were introduced into vessels with potential predators; the losses due to predation were recorded at intervals.

3. The results of these observations strongly suggest that barnacle nauplii lack a generalized defense against predation, and there was no indication that the frontolateral horns and horn glands are used as venomous organs. The observed indifference of certain hydromedusae to most contacts with barnacle nauplii suggests, however, that there might be a specialized defense mechanism.

4. Other possible functions of the frontolateral horns are discussed in view of the results.

LITERATURE CITED

Darwin, C., 1851. *A Monograph on the sub-class Cirripedia: The Lepadidae or pedunculated Cirripedes.* Ray Society, London, 400 pp.

Dohrn, A., 1870. Untersuchungen über Bau und Entwicklung der Arthropoden. 9. Eine neue Nauplius-Form (*Archizoëa gigas*). *Z. Wiss. Zool.,* **20**: 597–606.

Groom, T. T., 1894. On the early development of Cirripedia. *Phil. Trans. Roy. Soc. London Series B.,* **185**: 119–232.

Hardy, A. C., and R. Bainbridge, 1954. Experimental observations on the vertical migrations of plankton animals. *J. Mar. Biol. Ass. U.K.,* **33**: 409–448.

Kaufmann, R., 1965. Zur Embryonal- und Larvalentwicklung von *Scalpellum scalpellum* L. (Crust. Cirr.). *Z. Morphol. Tiere,* **55**: 161–232.

Krüger, P., 1940. Cirripedia, 3. Buch, Teil III, pp. 1–560. *In:* H. G. Bronns Klassen und Ordnungen des Tierreichs. Akademische Verlagsgesellschaft, Leipzig, 560 pp.

Lebour, M., 1920. The food of young fish. No. III. *J. Mar. Biol. Ass. U.K.* **12**: 261–324.

Lebour, M., 1922 The food of plankton organisms *J. Mar. Biol. Ass. U.K.,* **12**: 644–677.

Lebour, M., 1923. The food of plankton organisms. II. *J. Mar. Biol. Ass. U.K.,* **13**: 70–92.

Moyse, J., 1960. Mass rearing of barnacle cyprids in the laboratory *Nature,* **185**: 120.

Russell, F. S., 1953. *The Medusae of the British Isles.* Cambridge University Press, 530 pp.

Sanders, H. L., 1952. The herring (*Clupea harengus*) of Block Island Sound. *Bull. Bingham Oceanogr. Collect. Yale Univ.,* **13**: 220–237.

Wailes, G. H., 1936. Food of *Clupea pallasii* in Southern British Columbia waters. *Journal of the Biological Board of Canada,* **1**: 477–486.

Reference: *Biol. Bull.*, **138**: 219–234. (April, 1970)

TRANSITIONS BETWEEN HELICAL AND PROTOFIBRILLAR CONFIGURATIONS IN DOUBLET AND SINGLET MICROTUBULES IN SPERMATOZOA OF *STYLOCHUS ZEBRA* (TURBELLARIA, POLYCLADIDA)[1, 2]

MARY BETH THOMAS

Department of Zoology, University of North Carolina, Chapel Hill, North Carolina 27514

Microtubules occur widely in both plant and animal cells. They may exist either as singlets or doublets. The arrangement of microtubules within the cell is often precise, as exemplified by the 9 + 2 pattern of doublet and singlet microtubules in cilia, flagella and axial units (Fawcett and Porter, 1954), the double spiral of singlet microtubules in the axopodia of Heliozoa (Tilney and Porter, 1965), the parallel rows in the axostyle of certain flagellates (Grimstone and Cleveland, 1965), or the single rows of singlets which parallel the long axis of asymmetric cells (Byers and Porter, 1964) and nuclei (Burgos and Fawcett, 1955). Whether or not the component microtubules of these widely differing associations are functionally and/or structurally equivalent is not known. Similarities in size, appearance, and staining properties have been cited to suggest that cytoplasmic singlet microtubules and ciliary microtubules may be similar, and that these, in turn, are similar to spindle microtubules (for references, see Behnke and Forer, 1967). Turner (1968) has also concluded that centriolar triplets are identical with other types of microtubules described.

Biochemical studies tend to support this view, at least in part. The actin-like, nucleotide-containing protein described from the flagella of starfish spermatozoa (Plowman and Nelson, 1962) has been found to occur in the outer fibers of the flagella of sea urchin spermatozoa and in the cilia of *Tetrahymena*, and has been shown to contain a guanine nucleotide (Stephens, Renaud and Gibbons, 1967). The protein of the outer fibers has a molecular weight of *ca.* 59,000 in the flagella of sea urchin spermatozoa (Stephens, 1968) and 55,000 in cilia of *Tetrahymena* (Renaud, Rowe and Gibbons, 1968). Shelanski and Taylor (1967, 1968) have found a similar protein with a molecular weight of 60,000 in the central and outer fibers of sea urchin sperm flagella, and have demonstrated a guanine nucleotide binding site in both components of the flagella and a colchicine binding site in the central elements. The fact that a colchicine binding site has not been demonstrated by these workers in doublets has been attributed to the preparative methods used (Shelanski and Taylor, 1968).

The mitotic spindle apparatus has also been found to be composed of a protein similar to actin by Roslansky (Mazia, 1955), with a molecular weight of 66,000 ±

---

[1] Aided by a grant to D. P. Costello and Catherine Henley from the National Institutes of Health, GM 15311.

[2] Portion of a dissertation to be submitted to the faculty of the University of North Carolina, in partial fulfillment of the requirements for the Ph.D. degree.

7000 (Kane, 1967) and with a colchicine binding site (Borisy and Taylor, 1967). It would appear, then, that ciliary doublet and singlet microtubules and mitotic spindle microtubules are composed of similar subunits. No attempt has been made to analyze the composition of cytoplasmic microtubules, other than those constituting the spindle.

In spite of the similarities that have been shown to exist among microtubules, there is increasing evidence that all are not identical. Based on the effects of such physical and chemical agents as cold, heat, colchicine, and pepsin on the microtubules of cranefly and rat spermatozoa, and rat tracheal cilia, Behnke and Forer (1967) have described four classes of microtubules: (1) A-tubules of ciliary doublets; (2) B-tubules of ciliary doublets; (3) central and accessory tubules of cilia; and (4) cytoplasmic tubules and spindle tubules. This classification of the A- and B-tubules is in agreement with the demonstration that ATPase, in the form of dynein, is contained in the "arms" which are associated only with the A-tubule (Gibbons, 1963, 1965, 1966; Gibbons and Rowe, 1965). Also, differences in solubility have been shown to exist between the A- and B-tubules of *Chlamydomonas reinhardii* (Jacobs, Hopkins and Randall, 1968). The A- and B-tubules of the lungfluke spermatozoon are affected differently by treatment with pepsin (Burton, 1968), and the two subtubules stain differently with ethanolic phosphotungstic acid following fixation with osmium tetroxide (Gordon and Bensch, 1968). Furthermore, cytoplasmic singlets and spindle microtubules in many forms are susceptible to cold treatment and colchicine (Tilney and Porter, 1967; Tilney, 1968; Inoué and Sato, 1967), whereas ciliary microtubules are not (Burton, 1968).

Numerous studies suggest, however, that the four classes of microtubules described by Behnke and Forer may have to be further subdivided. Jacobs *et al.* (1968) have reported that the two central singlet ciliary microtubules of *Chlamydomonas* do not have identical solubilities. Allen (1968) has demonstrated in sectioned material the presence of arms on only one of the central tubules in cilia of *Tetrahymena,* and Chasey (1969) has found projections from only one of the central tubules in negatively stained preparations from the same material.

Furthermore, all cytoplasmic microtubules may not be identical. Behnke and Zelander (1967) have subdivided cytoplasmic microtubules into O-tubules (those preserved by osmium fixation) and G-tubules (those preserved by glutaraldehyde fixation, but not by osmium fixation) and have shown that G-tubules are more sensitive to certain solutions at a neutral pH than are O-tubules.

Burton (1966b) has found that the cortical singlet microtubules in spermatozoa of the lungfluke have a helical configuration, whereas cortical microtubules of a trypanosome show no evidence of this helical pattern. The spermatozoa of the lungfluke are unaffected by treatment with cold or colchicine, in this way resembling ciliary microtubules more than cytoplasmic ones (Burton, 1968). In addition, by use of the Markham rotation technique, cytoplasmic singlets of the lungfluke sperm have been estimated to be made up of 8 subunits per turn of the helix (Burton, 1966a), whereas 12 or 13 have been reported, using the same technique, in meristematic cells of *Juniper* (Ledbetter and Porter, 1964), in *Chlamydomonas* (Ringo, 1967), and in spermatozoa of *Sciara* (Phillips, 1966).

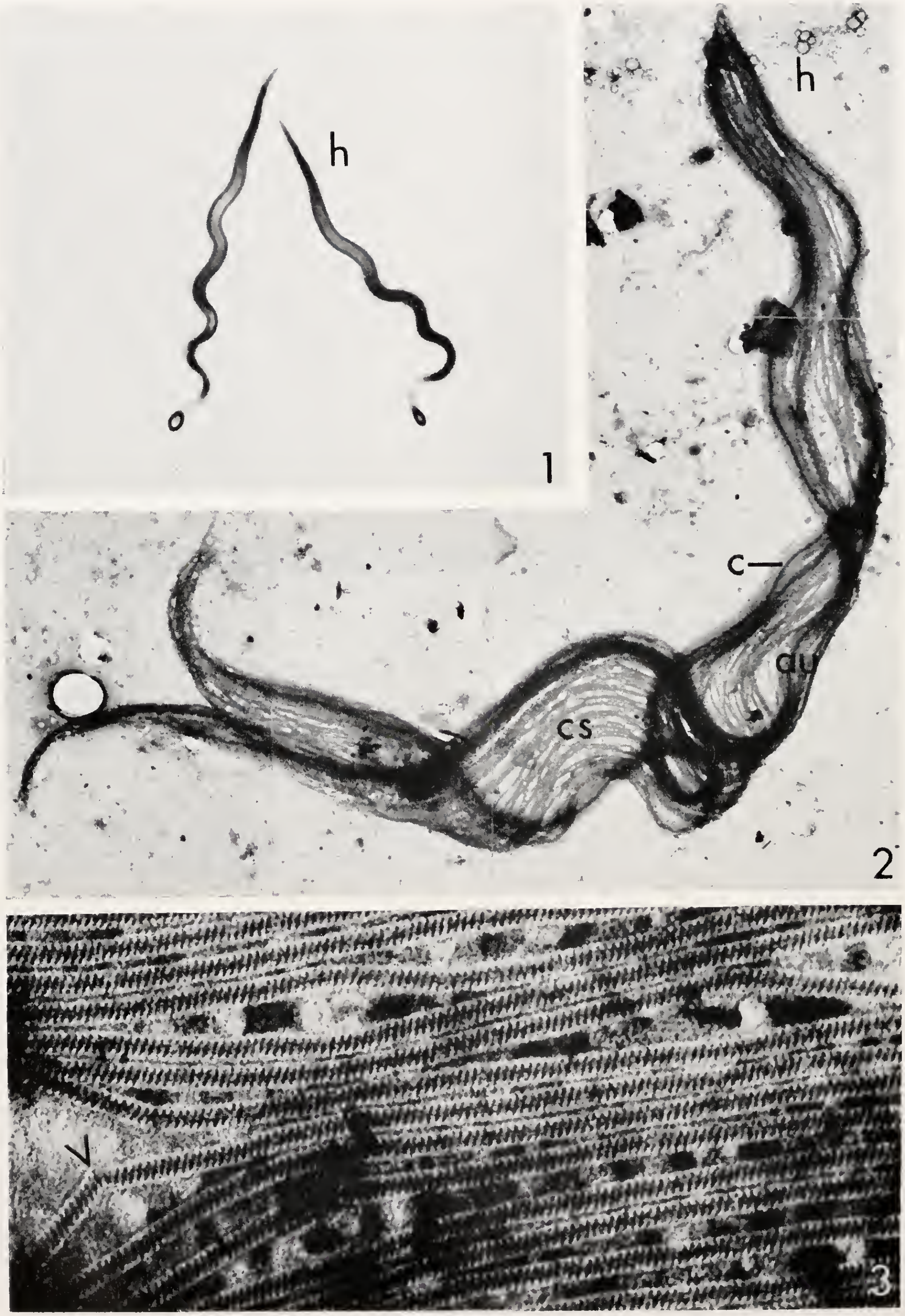
Perhaps the difference between the points of view that microtubules are identical and that they differ can be resolved by showing that some, if not all, microtubules are composed of similar subunits, and that the differences in the effects of various treatments result from differences in their macromolecular associations. That polymorphism can result from slight variations in the associations of similar subunits has been discussed by Casper (Satir, 1970). As a possible example, doublets and some singlets have been shown to exhibit both helical and filamentous configurations in negatively stained material. Cortical singlets with a helical configuration in lungfluke spermatozoa have been observed to undergo a transition from the helical to a filamentous configuration, in which approximately 5 beaded, longitudinal protofibrils occurred in negatively stained preparations (Burton, 1966b). Behnke and Zelander (1967) have observed protofibrils in microtubules of mammalian blood platelets. Protofibrils have been reported frequently for doublet microtubules negatively stained with phosphotungstic acid (Pease, 1963; André and Thiéry, 1963; Grimstone and Klug, 1966). Grimstone and Klug (1966) have shown by optical diffraction that intact doublets display definite longitudinal periodicities, and they interpret their results in terms of a helical surface lattice. A helical configuration in negatively stained doublet microtubules in spermatozoa of *Dugesia* is shown in a micrograph by Silveira (Porter, 1966). Pease (1963) has shown that the subfibers of doublets, when separated from one another, assume a helical configuration. André and Thiéry (1963) have demonstrated the presence of a helical pattern in the A-tubule of human spermatozoa. There is evidence, therefore, that doublets and some cytoplasmic singlets treated with phosphotungstic acid may exist in either a protofibrillar or a helical form.

In the present study, axonemal doublet and cortical singlet microtubules of the spermatozoa of the polyclad, *Stylochus zebra*, were negatively stained with phosphotungstic acid (PTA) (Brenner and Horne, 1959). The helical and protofibrillar configurations of these two classes of microtubules were compared in an attempt to ascertain whether or not the same subunits can exist in both configurations, in both types of microtubules.

Materials and Methods

Specimens of *Stylochus zebra*, obtained from the Supply Department of the Marine Biological Laboratory at Woods Hole, Massachusetts, were maintained in dishes of sea water in the laboratory at room temperature. Spermatozoa were removed from the animals by transecting the vas deferens with needles and applying pressure along the ducts to free the spermatozoa into a Columbia watchglass. Several drops of 1% PTA, adjusted to pH 6.8 with 0.1 N NaOH, were added, and the sperm quickly dispersed in the fluid with a pipette. A drop of the suspension was then transferred to a Formvar-carbon-coated 200 mesh copper grid for from 2 to 10 minutes. Excess fluid was drained from the grid with filter paper and the preparation was allowed to dry. This material was examined with a Zeiss 9A electron microscope.

Each sample of spermatozoa was observed by phase contrast microscopy before negative staining, to insure that all spermatozoa used were healthy, as determined by their morphology and motility. In one experiment, spermatozoa from an animal in an advanced state of deterioration were used for comparison with the sperm of healthy animals.



Figures 1–3.

OBSERVATIONS

The mature spermatozoa of *Stylochus zebra* are approximately 35 μ long and 1.25 μ in diameter. A straight head is continuous with a corkscrew-shaped tail; a fine filament projects anteriorly (Fig. 1). There are no free flagella, but two lateral axial units with the "9 + 1" pattern (Henley, Costello, Thomas and Newton, 1969) adhere to the tail, lying in two grooves (or compartments) just beneath the cell membrane. A single row of from 100 to 120 cortical singlet microtubules is arranged just beneath the plasma membrane of the spermatozoon. In negatively stained preparations, the different microtubular elements (cortical singlets, axonemal doublets, and the complex core of the axial unit) can be identified readily (Fig. 2). The present account will be limited to a consideration of the cortical singlets and axonemal doublets of the spermatozoa.

Cortical singlets

Following negative staining, the cortical singlets usually fall on the grid in parallel array, either in straight or smoothly curved rows. Sharp bends, when they occur, are accompanied by clean breaks in the tubules (Fig. 3). The most obvious feature of the singlets is the arrangement of the subunits to produce a striking helical configuration, the pitch of which varies from 12° to 24°. The angles observed most frequently are between 16° and 19°. The alternating electron-dense and electron-lucent bands which make up the helix are repeated at intervals of *ca.* 80 Å. The diameter of these helical tubules is 200 to 240 Å. The pattern described may be interrupted in two ways:

(1) The helical pattern may become altered in spermatozoa from deteriorating animals (Fig. 4). The tubule tends to maintain its integrity rather than to collapse. However, it becomes spread laterally to a diameter of *ca.* 290 Å and stretched longitudinally so that the distances between the gyres of the helices are increased by *ca.* 15%. The subunits become visible, and lateral and longitudinal linkages between these subunits can be seen in places. Five longitudinal protofibrils can be counted under these conditions. With further spreading complete collapse of the tubule is often observed; the helical structure is lost entirely and 7 or 8 protofibrils can be seen in a flat plane.

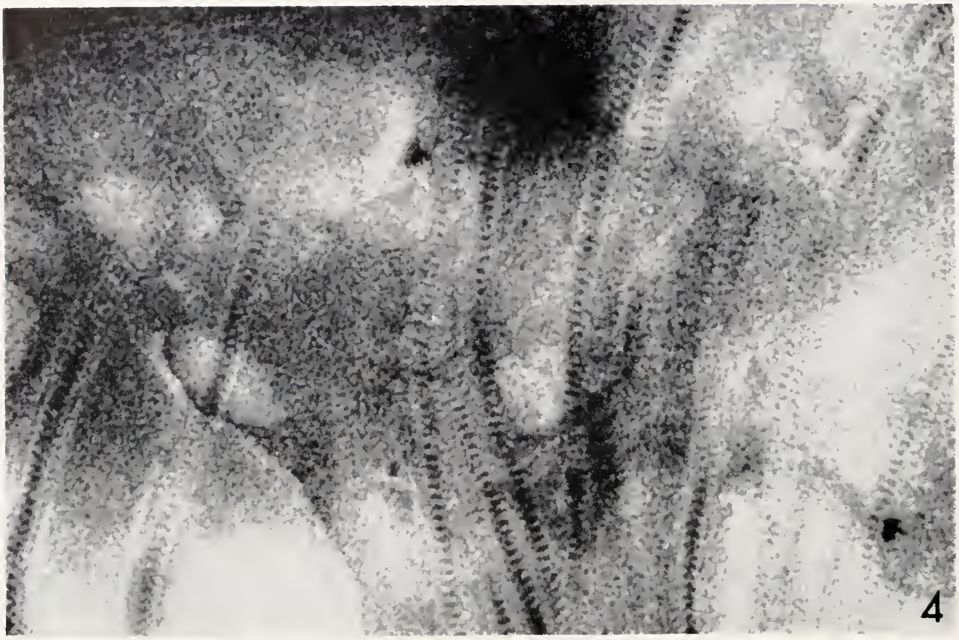
(2) The helical configuration may also be interrupted, even in apparently healthy spermatozoa, when large regions of several adjacent microtubules undergo a transition to a ribbon-like structure, usually 350 Å in diameter (Figs. 5 and 6). At high magnification, these regions are seen to be composed of 6 or 7 protofibrils made up of subunits which give the fibrils a beaded appearance (Fig. 7). The actual size of the subunits cannot be measured with accuracy in negatively stained material, but their center-to-center spacing, measured along the length of the

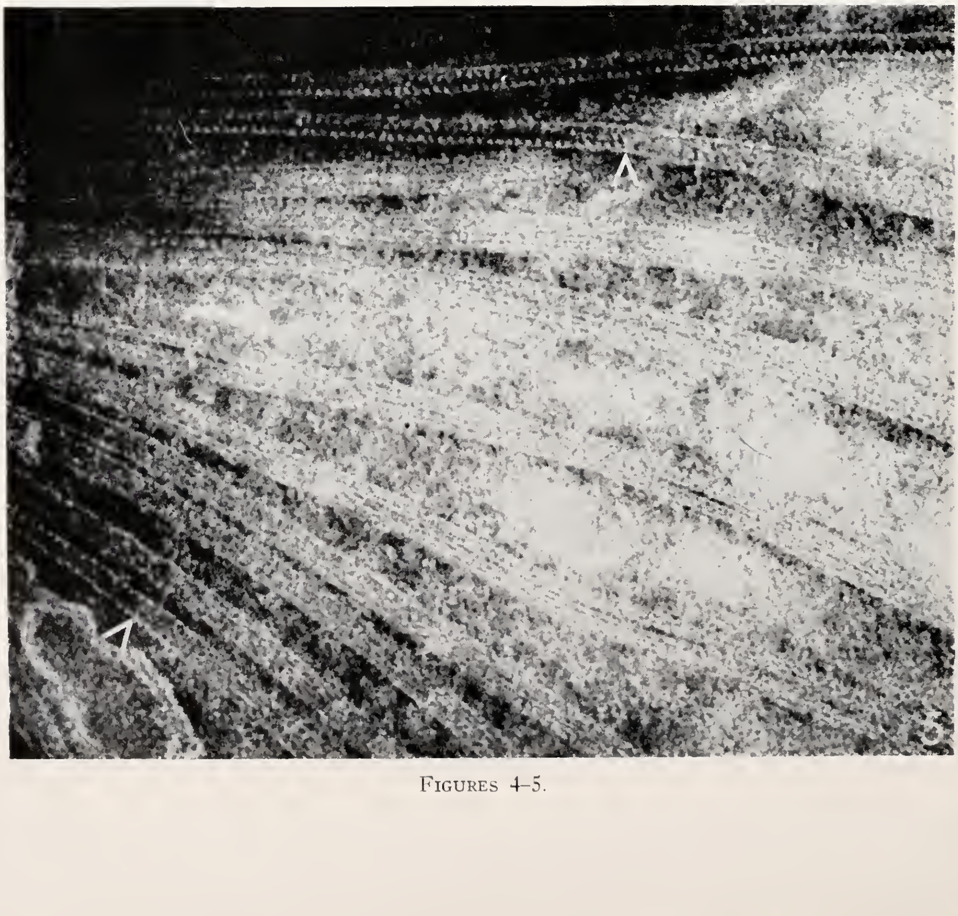
---

FIGURE 1. Mature spermatozoa of *Stylochus zebra* as observed by phase contrast microscopy. *h* = head. Magnification: 1600×.

FIGURE 2. Isolated microtubular complex of PTA-treated spermatozoon. Present are two 9 + 1 axonemes plus all the cortical singlets. *h* = head; *au* = axial unit; *c* = core of axial unit; *cs* = cortical singlet microtubules. Magnification: 7000×.

FIGURE 3. Cortical singlet microtubules with helical configuration revealed by negative staining. A break associated with a sharp bend is indicated by *V*. Magnification: 152,000×.





FIGURES 4–5.

protofibril, is *ca.* 40 to 45 Å. The lateral spacing of the protofibrils from center to center is *ca.* 50 Å, although at maximum spreading the distance may be much greater. These protofibrillar regions may continue as such until the microtubule ends distally, or may undergo a transition back to the helical form.

Axonemal doublets

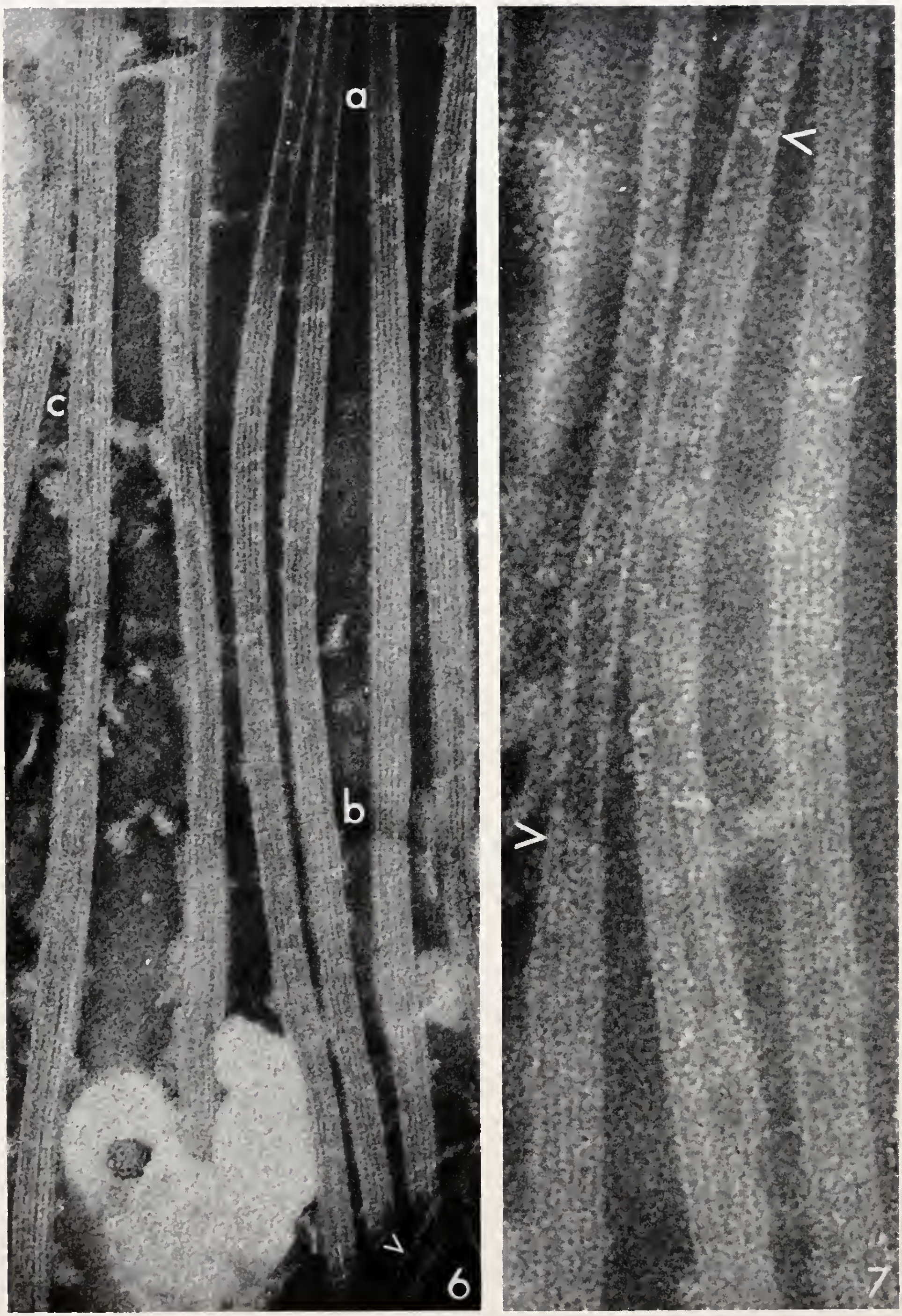
The 9 doublets of each axial unit, negatively stained, are approximately 440 to 480 Å in diameter in the relatively unmacerated state, although with increased maceration by PTA the diameter may be greater than 600 Å, especially in microtubules of degenerating spermatozoa (Fig. 11). Intact, negatively stained doublets show electron-lucent boundaries laterally and the two subtubules are separated by a median electron-lucent area *ca.* 80 Å wide (Fig. 8). In this material, even in tubules that have been macerated for only a short time, protofibrils can usually be seen. The protofibrils are made up of subunits which are spaced *ca.* 40 to 45 Å center-to-center along their lengths (Figs. 9 and 10). The lateral spacing of the protofibrils is relatively constant at *ca.* 50 Å, although in localized regions they may become more widely separated. With increased maceration, the electron-lucent median area, interpreted as the central partition separating the two subtubules, appears to split longitudinally into two protofibrils which often show cross-connections between them (Fig. 9). These protofibrils remain more electron-lucent than the other protofibrils, suggesting that they may represent two or more protofibrils superimposed on one another. The same is true for the protofibrils that occur laterally in each subtubule (Figs. 8 and 9). Occasionally one subtubule is completely lost, in which case the double set of protofibrils of the median wall usually remains (Fig. 9). Short regions of the doublet may also be seen to be turned on edge, in which case the diameter is the same as that of a single subtubule (Fig. 8).

In most cases in the PTA-macerated material, both subtubules of the doublet end distally at the same level. Occasionally, however, one subtubule drops out and the other continues as a single unit for as much as 2.5 μ, although the length of the remaining subtubule is usually only 1.5 to 2.0 μ (Fig. 14). Figure 13 shows that the disappearance of the shorter tubule results from its termination, rather than from rotation of the doublet. The electron-lucent protofibrils, interpreted to be the wall between the doublets, continue as part of the remaining tubule, maintaining their recognizable appearance for a short distance (Figs. 12, 13, and 14). The diameter of the remaining tubule is that of one subtubule including the common wall. At a distance of from 400 to 850 mμ from the point at which the first tubule drops out, the configuration of the remaining tubule changes abruptly into a helical pattern which is maintained for distances of up to 900 mμ. Regardless of the variation in the diameter of the tubule proximal to the helical portion, due to spreading, the diameter of the helix is from 250 to 260 Å. The center-to-center spacing between gyres is 80 Å and the pitch is usually 18° to 20°, but may vary

---

FIGURE 4. Cortical singlet microtubules of negatively stained spermatozoa from deteriorating animal. The microtubules are spread both laterally and longitudinally, and only traces of the original helical organization remain. Magnification: 152,400 ×.

FIGURE 5. Helical and protofibrillar configuration in cortical singlets of spermatozoa from healthy animals. Transitions are noted by *V*'s. Seven protofibrils can be counted in some regions, although six appear more frequently. Magnification: 152,000 ×.



FIGURES 6–7.

from 15° to 21° (Fig. 15). In a few instances the helix is interrupted for a short distance, beyond which the direction of the helix is reversed. This reversal is attributed to staining of opposite sides of the helix, rather than to an actual reversal in the direction of coiling.

The helical configuration usually does not continue to the end of the tubule; rather, the protofibrillar arrangement is observed at the terminus (Figs. 14 and 15). Six protofibrils can sometimes be counted at the extreme distal end.

A summary of the properties of the doublet and singlet microtubules is given below:

	Cortical singlet	Doublet subtubule
Diameter of helical tubule	200–240 Å	250–260 Å
Diameter of filamentous tubule	350 Å	260–400 Å
Gyres of helix (center-to-center)	80 Å	80 Å
Pitch of helix	16–19°	18–20°
Subunit spacing in protofibrils:		
Longitudinally (center-to-center)	40–45 Å	40–45 Å
Laterally (center-to-center)	50 Å or more	50 Å or more
Number of protofibrils/tubule	6–7	5–6

Discussion

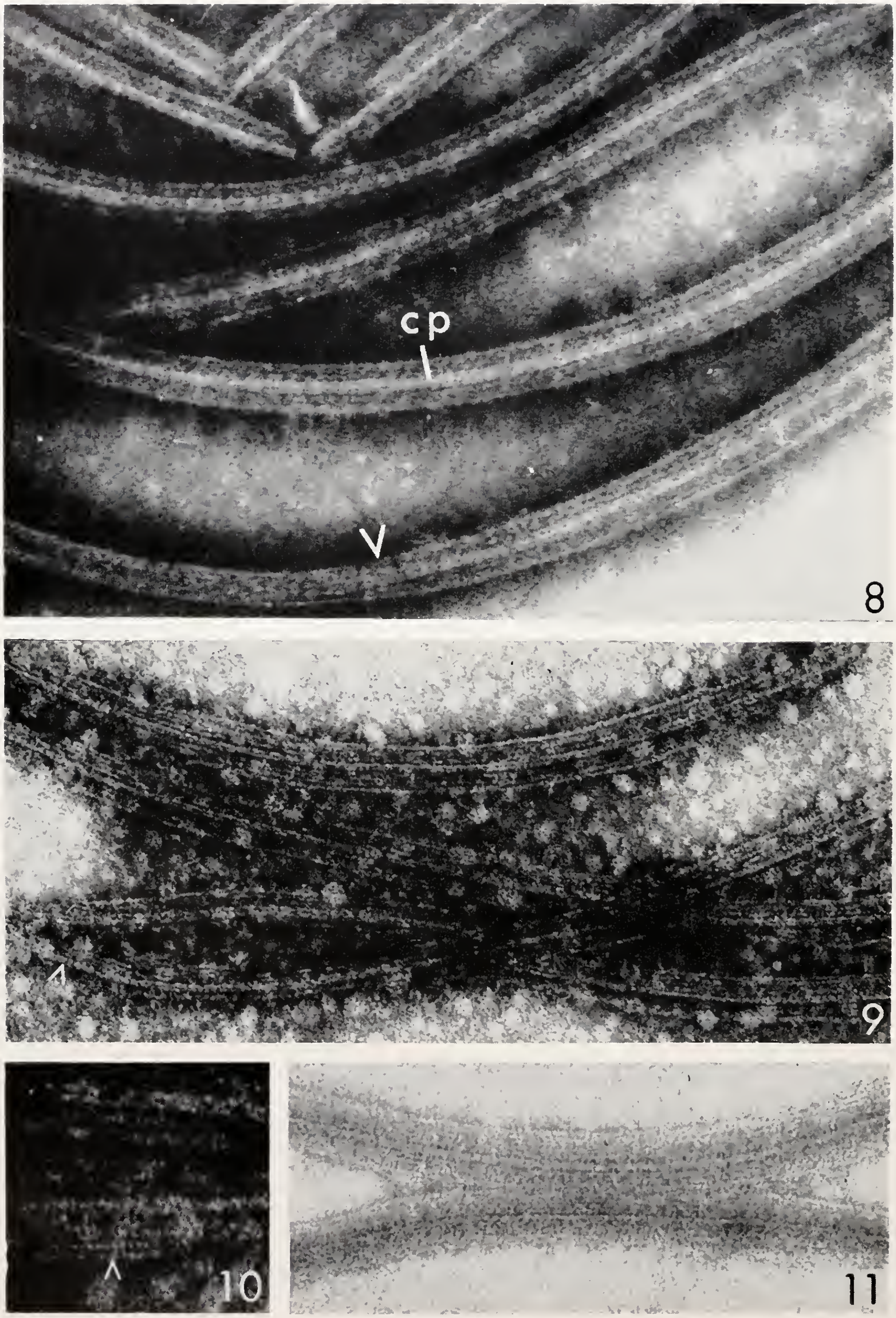
Both the cortical singlets and at least one subtubule of axonemal doublets of the spermatozoa of *Stylochus* can assume a helical or protofibrillar configuration under certain conditions of maceration and negative staining with PTA. So far, in our experience, the doublet subtubule has been seen to acquire the helical pattern only at some distance from the point at which the other member of the doublet drops out. Other reports of the occurrence in doublet microtubules of the helical structure described are those by Pease (1963), André and Thiéry (1963) and Porter (1966). Pease showed a helical structure in subtubules that had been completely separated from one another. André and Thiéry demonstrated the helical pattern in one member of the doublet only, the A-tubule according to their interpretation. A micrograph by Silveira (Porter, 1966) of doublets from the spermatozoa of *Dugesia* shows a helical structure for one, or perhaps both, of the members of the doublet. The suggestion from these studies and from the observations reported here is that the subunits of at least one doublet subtubule tend in PTA to acquire a helical configuration similar to that of the cortical singlets of the spermatozoa of the lungfluke (Burton, 1966a, 1966b, 1968) and of *Stylochus*. This may indicate that basically similar subunits and bonds are involved in both classes of microtubules.

A comparison of the helical structure of the doublet subtubule with that of the cortical singlet reveals that within the limitations of error in measurement, the two

---

FIGURE 6. Cortical singlet microtubules showing varying degrees of separation of protofibrils. A region of transition from the helical to the protofibrillar condition is indicated by the *V* at the lower right. Tubules at *a* appear intact, but show no evidence of the helical pattern. Four or 5 protofibrils can be seen. With further separation, as in region *b*, 6 or 7 protofibrils can be counted. Equidistant lateral spacing of these protofibrils suggests the presence of lateral bonding. At *c* the protofibrils have completely separated in some regions. Magnification: 152,400 ×.

FIGURE 7. Transitions between helical and protofibrillar configurations in cortical singlets. The points at which the transitions occur are indicated by *V*'s. Magnification: 304,800 ×.



Figures 8–11.

types of microtubules differ in gross structure only in the diameters of the helices. If the differences in diameter would allow for the presence of 8 subunits per gyre in the cortical singlets (Burton, 1966a) and 12 or 13 subunits in the doublet subtubule (Ledbetter and Porter, 1964; Philips, 1966; Ringo, 1967), the numbers of subunits reported to compose each class of microtubules, then the contention that both classes of tubules are composed of equivalent subunits would be considerably strengthened. However, using the measurements presented here, the maximum number of subunits by which the two classes of tubules can differ appears to be two, which is two or three fewer than the number required. It is necessary to conclude, therefore, that either (1) the number of subunits in one or both classes of microtubules in spermatozoa of *Stylochus* differs from that reported for other organisms, or (2) the subunits are not equivalent in the two classes of microtubules. At this time it is not possible to decide between the two alternatives.

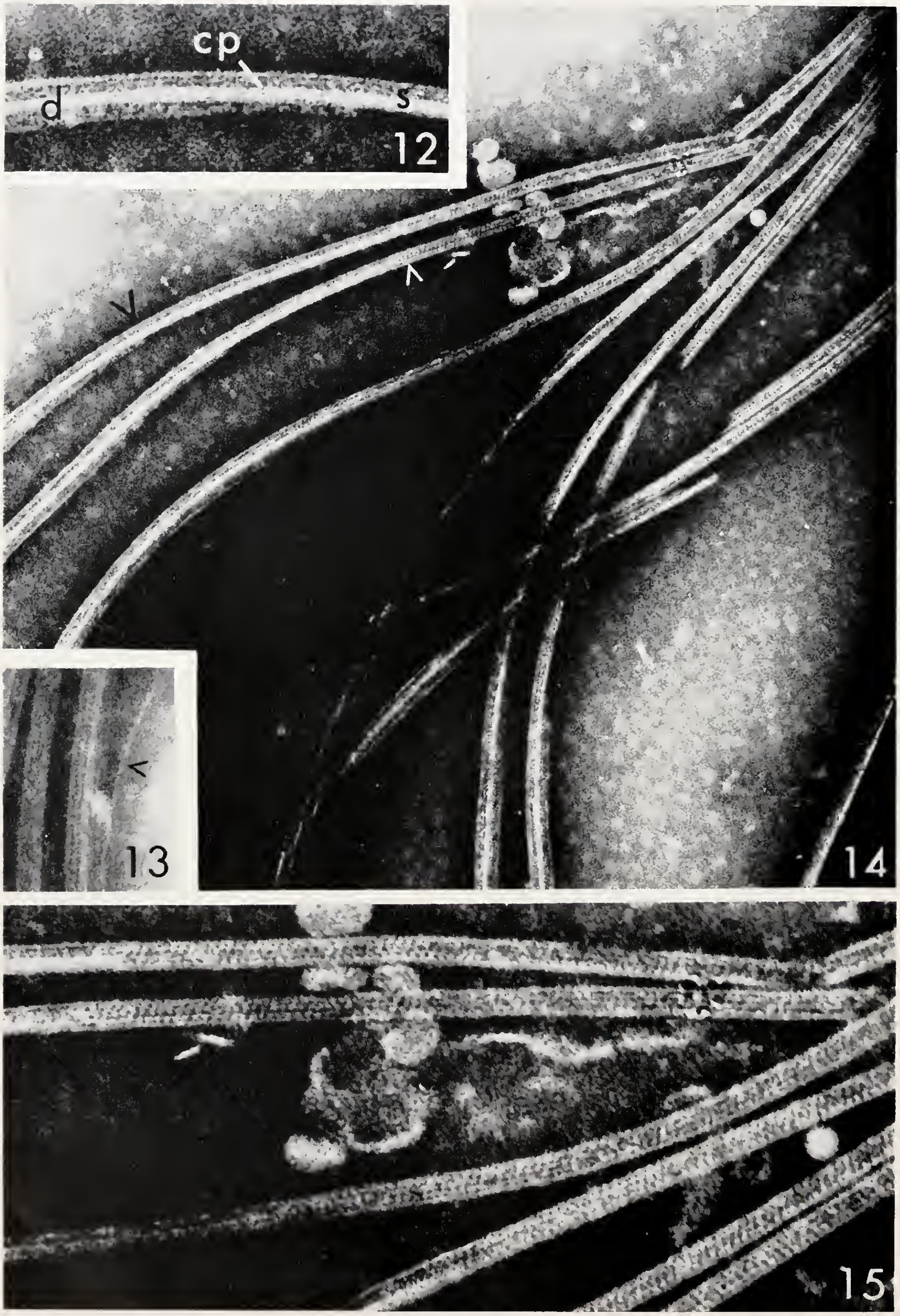
Examination of the fate of the common wall of the doublet at the distal end of the axial unit in negatively stained material may give some information about the relationship of that wall to each of the two subtubules. It has not been shown conclusively whether the wall belongs wholly to one subtubule, or whether it is equally shared by both members. Ringo (1967) has interpreted the wall as being common to both subtubules in the flagella of *Chlamydomonas*. In the sectioned material of Phillips (1966) the wall separating the two tubules of the axial unit of spermatozoa of *Sciara* is thickened; the author suggests that there may be some interpenetration of the walls of the two members of the doublet. In the same paper, however, he shows that in the spermatozoa of *Hippodamia* and *Draeculacephala* the two subtubules of the doublet separate at the peripheral end; in sectioned material it can be seen that the A-subtubule is circular in cross-section, while the B-subtubule is C-shaped. Grimstone and Klug (1966) discuss the relationship of the wall to the two tubules in negatively stained material. They show that in relatively unmacerated doublets the common wall appears as a single line. Increased maceration results in a widening of the area and the appearance of two lines. Grimstone and Klug suggest that when the two lines appear, the subtubules have essentially separated from one another, and point out that when this occurs, both members of the doublet appear to be intact. Yet they state that after separation one subtubule collapses more readily than the other, indicating that the wall of one tubule may be incomplete. Since Grimstone and Klug suggest that upon separation of the doublet one member of the pair of "white lines" was contained in each subtubule, producing two presumably intact tubules, it is interesting that in *Stylochus* when one subtubule drops out, either along the length of the doublet or at the distal end, both lines representing the common wall continue along the

FIGURE 8. Doublet microtubules showing protofibrils and the electron-lucent central partition. The *V* marks a region in which one of the doublets is turned on edge. *cp* = central partition. Magnification: 152,400 ×.

FIGURE 9. Doublet microtubules showing 10 or 11 protofibrils. At *V* one subtubule has been digested away; the central partition maintains an association with the remaining subtubule. Note the cross-connections between the fibrils of the central partition. Magnification: 152,400 ×

FIGURE 10. Subunits of protofibrils of doublet microtubule. Magnification: 304,800 ×.

FIGURE 11. Two doublet microtubules of a spermatozoon from a deteriorating animal. Magnification: 152,400 ×.



FIGURES 12–15.

remaining subtubule. This may suggest that even in the intact doublets the common wall is more closely associated with one subtubule than the other.

It is not certain if the transformation at the distal end from doublet to singlet by loss of one member of the pair occurs naturally or results from the maceration by PTA. Since maceration by PTA has been observed to proceed from the distal to the proximal end of the axonemal complex (Costello, Henley and Ault, 1969), what is observed to be the termination of the doublets may be some distance proximal to the actual end of the doublets, due to the action of the PTA. If this is the case, and if one tubule is more sensitive to maceration (Grimstone and Klug, 1966), then the difference in the lengths of the two tubules may result entirely from the treatment. On the other hand, Satir has observed that in cilia of the mussel, the A-subtubule is consistently longer than the B-subtubule (Satir, 1967, 1968). Therefore, the difference in length reported here could reflect the situation in the normal axoneme. Some credence is given to this possibility by the fact that when one subtubule does remain, all 9 doublets show the phenomenon.

The significance of the existence of protofibrillar and helical forms in negatively stained material to the normal function of the microtubules is not apparent. Neither the helical nor the protofibrillar arrangement seems to be characteristic of the intact doublet, since negatively stained, unmacerated doublets rarely show either pattern, and helical configurations comparable to those shown here have never been demonstrated in sectioned material. The arrangement of the subunits may be far more complex, especially in the light of the 40, 80, 160 and 480 Å longitudinal periodicities described by Grimstone and Klug (1966) and others. The cortical singlets, however, appear to exist in the helical form in the untreated condition, since helices have been demonstrated in sectioned material (Burton, 1966a) and since they are present even in the earliest stages of PTA maceration. The natural occurrence of transitions to the protofibrillar condition is much more doubtful, however, since its occurrence is relatively infrequent and its location along the microtubules is unpredictable. In addition, it can be related to the extent of the PTA treatment.

It should be mentioned, however, that the transition from the helical to the filamentous condition described here is observed frequently enough to suggest that it is the result of the treatment, rather than of natural disintegration processes in

---

FIGURE 12. Doublet microtubules near distal end of axial unit at the point at which one subtubule terminates. Note that the electron-lucent central partition continues along the remaining subtubule. This figure is an enlargement of the region near the black *V* in Figure 14. *d* = doublet microtubule; *s* = subtubule of doublet; *cp* = central partition. Magnification: 152,000 ×.

FIGURE 13. Portion of doublet microtubule at the point at which one subtubule disappears. The position and obvious termination of the protofibril denoted by the *V* show that the disappearance of the subtubule results from its termination, rather than from rotation of the doublet. Note that the central partition remains associated with the continuing subtubule. Magnification: 152,000 ×

FIGURE 14. Region near the distal end of an axial unit, showing the disappearance of one subtubule (black *V*) and the transition to the helical configuration in the remaining subtubule (white *V*). Note that in the subtubule denoted by the white *V* at least 4 protofibrils can be seen at the terminus. Magnification: 76,000 ×.

FIGURE 15. Enlargement of portion of Figure 14, showing the transitions to the helical configuration in one subtubule of several doublets. Four or 5 protofibrils can be seen at the terminus. Magnification: 152,000 ×

the spermatozoon, as described by Burton (1966b). Since the spermatozoa were removed directly from the vas deferens, the possible presence of degenerating spermatozoa from the seminal receptacle was virtually eliminated. In the experiment in which spermatozoa from deteriorating animals were negatively stained, transitions from helices to protofibrils were observed to occur more frequently; however, both doublet and singlet microtubules of degenerating spermatozoa have a characteristic "fuzzy" appearance, and the gyres of the helical singlets are spread apart and are irregular in outline. The transition to the protofibrillar arrangement can be seen in spermatozoa which show none of the irregularities characteristic of the degenerating spermatozoa.

I wish to thank Dr. Donald P. Costello and Dr. Catherine Henley for their suggestion and direction of the problem. Their constructive criticism and constant encouragement are gratefully acknowledged. I would also like to thank Miss Nancy C. Edwards for her help in the preparation of the typescript.

SUMMARY

1. In spermatozoa of *Stylochus zebra* negatively stained with phosphotungstic acid, both cortical singlet and axonemal doublet microtubules can exist in a protofibrillar or a helical configuration. In cortical singlets the helical configuration appears more frequently; transitions to the protofibrillar condition occur at random and appear to result from the treatment with PTA. In doublet microtubules, the protofibrillar arrangement is the usual one. The helical structure is encountered only at the distal end of the axial unit in the single subtubule that persists distal to the termination of the other member of the doublet.

2. Comparison of the dimensions of protofibrillar and helical configurations in both singlets and doublets reveals that the two types of microtubules are similar. The observation that each type of microtubule can assume the characteristic configuration of the other type suggests that the structural differences of the two classes of microtubules result from alternative associations of equivalent subunits.

3. The central partition between the two subtubules of a doublet appears to be more closely associated with one of the two subtubules, since it remains when one subtubule drops out, either along the length of the doublet or at its distal end.

LITERATURE CITED

Allen, R. D., 1968. A reinvestigation of cross-sections of cilia. *J. Cell Biol.*, **37**: 825–831.

André, J., and J.-P. Thiéry, 1963. Mise en évidence d'une sous-structure fibrillaire dans les filaments axonématiques des flagelles. *J. Microsc.*, **2**: 71–80.

Behnke, O., and A. Forer, 1967. Evidence for four classes of microtubules in individual cells. *J. Cell Sci.*, **5**: 169–192.

Behnke, O., and T. Zelander, 1967. Filamentous substructure of microtubules of the marginal bundle of mammalian blood platelets. *J. Ultrastruct. Res.*, **19**: 147–165.

Borisy, G. G., and E. W. Taylor, 1967. The mechanism of action of colchicine. Colchicine binding to sea urchin eggs and the mitotic apparatus. *J. Cell Biol.*, **34**: 525–548.

Brenner, S., and R. W. Horne, 1959. A negative staining method for high resolution microscopy of viruses. *Biochim. Biophys. Acta*, **34**: 103–110.

Burgos, M. H., and D. W. Fawcett, 1955. Studies on the fine structure of the mammalian testis; I. Differentiation of the spermatids in the cat (*Felis domestica*). *J. Biophys. Biochem. Cytol.,* **1**: 287–300.

Burton, P. R., 1966a. Substructure of certain cytoplasmic microtubules: An electron microscope study. *Science,* **154**: 903–905.

Burton, P. R., 1966b. A comparative electron microscope study of cytoplasmic microtubules and axial unit tubules in a spermatozoon and a protozoan. *J. Morphol.,* **120**: 397–424.

Burton, P. R., 1968. Effects of various treatments on microtubules and axial units of lung-fluke spermatozoa. *Zeitschr. & Zellforsch.,* **87**: 226–248.

Byers, B., and K. R. Porter, 1964. Oriented microtubules in elongating cells of the developing lens rudiment after induction. *Proc. Nat. Acad. Sci.,* **52**: 1091–1099.

Chasey, D., 1969. Observations on the central pair of microtubules from the cilia of *Tetrahymena pyriformis. J. Cell Sci.,* **5**: 453–458.

Costello, D. P., C. Henley and C. R. Ault, 1969. Microtubules in spermatozoa of *Childia* (Turbellaria, Acoela) revealed by negative staining. *Science,* **163**: 678–679.

Fawcett, D. W., and K. R. Porter, 1954. A study of the fine structure of ciliated epithelia. *J. Morphol.,* **94**: 221–281.

Gibbons, I. R., 1963. Studies on the protein components of cilia from *Tetrahymena pyriformis. Proc. Nat. Acad. Sci.,* **50**: 1002–1010.

Gibbons, I. R., 1965. Chemical dissection of cilia. *Arch. Biol.,* **76**: 317–352.

Gibbons, I. R., 1966. Studies of the adenosine triphosphatase activity of 14S and 30S dynein from cilia of *Tetrahymena. J. Biol. Chem.,* **241**: 5590–5596.

Gibbons, I. R., and A. J. Rowe, 1965. Dynein: A protein with adenosine triphosphatase activity from cilia. *Science,* **149**: 424–426.

Gordon, M., and K. G. Bensch, 1968. Cytochemical differentiation of the guinea pig sperm flagellum with phosphotungstic acid. *J. Ultrastruct. Res.,* **24**: 33–50.

Grimstone, A. V., and L. R. Cleveland, 1965. The fine structure and function of the contractile axostyles of certain flagellates. *J. Cell Biol.,* **24**: 387–400.

Grimstone, A. V., and A. Klug, 1966. Observations on the substructure of flagellar fibres. *J. Cell Sci.,* **1**: 351–362.

Henley, C., D. P. Costello, M. B. Thomas and W. D. Newton, 1969. The "9 + 1" pattern of microtubules in spermatozoa of *Mesostoma* (Platyhelminthes, Turbellaria). *Proc. Nat. Acad. Sci.,* **64**: 849–856.

Inoué, S., and H. Sato, 1967. Cell motility by labile association of molecules. *J. Gen. Physiol.,* **50**: 259–288.

Jacobs, M., J. Hopkins and J. Randall, 1968. The flagellar proteins of a paralyzed mutant of *Chlamydomonas reinhardii. J. Cell Biol.,* **39**: 66a.

Kane, R. E., 1967. The mitotic apparatus. Identification of the major soluble component of the glycol-isolated mitotic apparatus. *J. Cell Biol.,* **32**: 243–253.

Ledbetter, M. C., and K. R. Porter, 1964. Morphology of microtubules of plant cells. *Science,* **144**: 872–874.

Mazia, D., 1955. The organization of the mitotic apparatus. *Symp. Soc. Exp. Biol.,* **9**: 335–357.

Pease, D. C., 1963. The ultrastructure of flagellar fibrils. *J. Cell Biol.,* **18**: 313–326.

Phillips, D. M., 1966. Substructure of flagellar tubules. *J. Cell Biol.,* **31**: 635–643.

Plowman, K. M., and L. Nelson, 1962. An actin-like protein isolated from starfish sperm. *Biol. Bull.,* **123**: 478.

Porter, K. R., 1966. Cytoplasmic microtubules and their functions, pp. 308–345. *In:* G. E. W. Wolstenholme, Ed., *Principles of Biomolecular Organization.* Little, Brown and Co., Boston.

Renaud, F. L., A. J. Rowe and I. R. Gibbons, 1968. Some properties of the protein forming the outer fibers of cilia. *J. Cell Biol.,* **36**: 79–90.

Ringo, D. L., 1967. The arrangement of subunits in flagellar fibers. *J. Ultrastruct. Res.,* **17**: 266–277.

Satir, B., 1970. Control of form in cells. *Science,* **167**: 307–309.

Satir, P., 1967. Morphological aspects of ciliary motility. *J. Gen. Physiol.,* **50**: 241–258.

Satir, P., 1968. Studies on cilia. III. Further studies on the cilium tip and a "sliding filament" model of ciliary motility. *J. Cell Biol.,* **39**: 77–94.

SHELANSKI, M. L., AND E. W. TAYLOR, 1967. Isolation of a protein subunit from microtubules. *J. Cell Biol.,* **34**: 549–554.

SHELANSKI, M. L., AND E. W. TAYLOR, 1968. Properties of the protein subunit of central-pair and outer-doublet microtubules of sea urchin flagella. *J. Cell Biol.,* **38**: 304–315.

STEPHENS, R. E., 1968. On the structural protein of flagellar outer fibers. *J. Mol. Biol.,* **32**: 277–283.

STEPHENS, R. E., F. L. RENAUD AND I. R. GIBBONS, 1967. Guanine nucleotide associated with the protein of the outer fibers of flagella and cilia. *Science,* **156**: 1606–1608.

TILNEY, L. G., 1968. Studies on the microtubules in Heliozoa. IV. The effect of colchicine on the formation and maintenance of the axopodia and the redevelopment of pattern in *Actinosphaerium nucleofilum* (Barrett). *J. Cell Sci.,* **3**: 549–562.

TILNEY, L. G., AND K. R. PORTER, 1965. Studies on the microtubules in Heliozoa. I. Fine structure of *Actinosphaerium* with particular reference to axial rod structure. *Protoplasma,* **60**: 317–324.

TILNEY, L. G., AND K. R. PORTER, 1967. Studies on the microtubules in *Heliozoa.* II. The effect of low temperature on these structures in the formation and maintenance of the axopodia. *J. Cell Biol.,* **34**: 327–343.

TURNER, F. R., 1968. An ultrastructural study of plant spermatogenesis. *J. Cell Biol.,* **37**: 370–393.

Reference: *Biol. Bull.*, **138**: 235–244. (April, 1970)

OXYGEN POISONING IN THE ANNELID *TUBIFEX TUBIFEX*
I. RESPONSE TO OXYGEN EXPOSURE[1]

JOANNE G. WALKER[2]

Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 81801

The freshwater Annelid *Tubifex tubifex* lives partially submerged in the bottom mud of ponds and slow-moving streams where the concentration of dissolved oxygen may become very low. During periods when oxygen is limited, these worms extend their tails far out of the mud and move with vigorous stirring motions to provide a more oxygen-rich environment (Alsterberg, 1922).

T. tubifex has no specialized respiratory system and is dependent on diffusion through the body surface for its oxygen supply. While *T. tubifex* is able to live in environments with extremely low oxygen tensions, it is also capable of maintaining a constant oxygen consumption when the oxygen tension is increased above atmospheric levels even to concentrations as great as one atmosphere oxygen pressure (Harnisch, 1935; Koenen, 1951; Walker, 1955). Anderson (1956) found that exposure to one atmosphere oxygen pressure for ten days is lethal to *T. tubifex* and also inhibits regeneration; 10^{-5} M cyanide stimulates some phases of regeneration. When regenerating worms are exposed to one atmosphere oxygen pressure in the presence of cyanide, survival is enhanced and the inhibition of regeneration by oxygen is partially reversed (Anderson, 1956).

The blood of *T. tubifex* contains hemoglobin in solution which has an extremely high affinity for oxygen (Fox, 1945). The role of hemoglobin in this organism is a matter for conjecture. Manwell (1959) has suggested that it may function to prevent oxygen poisoning in organisms such as *T. tubifex* which normally function at very low internal partial pressures of oxygen by protecting against development of high internal oxygen concentrations. Palmer (1968) on the other hand, has evidence that hemoglobin does function in oxygen transport but only when the external oxygen concentration falls below the critical tension.

In recent years reports have appeared of the effects of high oxygen pressure on subcellular organelles and upon electron transport systems both *in vitro* (Chance, Jamieson and Coles, 1965; Chance, Jamieson and Williamson, 1966; Jamieson and Chance, 1966) and *in vivo* (Chance *et al.*, 1965; Chance *et al.*, 1966). Whether the short-term reversible changes observed in these systems are related to the longer-term irreversible effects of oxygen in the whole organism is not readily apparent. It was therefore of interest to examine some of the factors influencing the pattern of oxygen poisoning in a whole organism which would be readily amenable to environmental manipulations. Because *T. tubifex* has considerable capacity for altering its metabolic patterns and responses in various environmental and physiological conditions, it appeared to be an interesting organism to use for such studies.

---

[1] These data are taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Illinois, Urbana, Illinois.

[2] Present address: Department of Veterinary Pathology, Ohio State University, 1925 Coffey Rd., Columbus, Ohio 43210.

MATERIALS AND METHODS

Worms were collected from a local drainage ditch and were stored covered with water in the stream bottom mud in a pail in the laboratory. The water used in this study was tap water which had been passed through activated charcoal filters. During initial experiments some difficulties were encountered in maintaining the worms for long periods of time. Contamination of the tap water with metal ions such as copper known to be toxic to *T. tubifex* (Jones, 1938) was suspected, therefore 0.05 g of the metal chelator disodium dihydrogen ethylenediamine tetraacetic acid (Hach Chemical Co.) per liter was added to all water used in these experiments. Immediately before use the worms were separated from the mud and each worm was anesthetized in 0.2% chloretone and examined under a compound microscope for species, absence of injury, lack of external parasites and state of regeneration. Two species of Oligochaeta were usually found together in the same collection. *T. tubifex* was identified during the microscopic examination by the setae pattern; four to five prominent capilliform setae together with usually six pectinate setae in the dorsal bundles of segments anterior to the clitellum and bifurcate setae only in the ventral bundles. The other species had short, bifurcate setae only in both the dorsal and ventral bundles. These organisms were identified as belonging to the genus *Limnodrilus*. Both normal and regenerating worms were included in the initial experimental series. All worms used in later experiments were cut for regeneration during the examination period in chloretone in order to ensure a more uniform experimental population. Each worm was placed into five ml water in an individual depression drilled in a block of household-type USP paraffin (Parawax, Standard Oil Co.); each worm was therefore isolated during the entire experimental procedure. This technique expedited the scoring of post-exposure responses to oxygen and eliminated the possibility of effects of contamination from other injured worms.

The chamber used for oxygen exposure was built of steel pipe and fitted with gas inlet and outlet valves and a pressure gauge. The chamber was flushed with oxygen (Linde, USP) for five minutes prior to increasing the pressure to the exposure conditions of four atmospheres absolute. When the effect of pressure alone was tested, the chamber was not flushed and nitrogen (Linde, high purity, dry) was added to the atmosphere of air already present until four atmospheres absolute were attained. All exposures were made at four atmospheres absolute; the dose was varied by varying the time of gas exposure. No difference in worm survival could be correlated with short or long periods of decompression, but after the initial experimental series a standard decompression of two pounds per minute was used.

Worms were exposed to oxygen in the paraffin blocks. After gas exposure the blocks were stored on the laboratory table under loosely fitting bell jars to prevent contamination and retard evaporation. Worms were examined several times daily for the first two or three days following oxygen exposure; afterwards, they were examined approximately once daily until at least 12 days had elapsed. The time intervals presented in the tables were chosen to correspond as closely as possible to times at which the actual observations were made. When observations were not made at these particular times, the data were obtained by graphic interpolation from the actual survival data immediately before and after the time given.

When the worms were exposed to interrupted oxygen doses, they were returned to atmospheric conditions during the interruption. The duration of interruption was taken as the time from the end of the first decompression until pressure was attained for the second oxygen exposure. Post-exposure survival was scored at various intervals measured from the end of the second decompression.

RESULTS

1. *Response of* T. tubifex *to four atmospheres absolute oxygen pressure*

A. *Gross appearance of oxygen-treated worms.* Worm behavor on removal from the oxygen chamber was closely related to the duration of oxygen exposure. The first noticeable response to oxygen was marked hyperactivity (one-hour exposure). The worms were extended and moving with vigorous stirring motions. Worm hyperactivity changed to a slower occasional whip-like thrashing for oxygen exposures of two to four hours. Following five- and six-hour exposures the worms were moving slowly and some were in a partially coiled or contracted state. After eight hours of oxygen treatment all worms contracted showing little movement. Any occasional movements were tortuous. After nine hours the first damage was apparent. Following longer oxygen exposures, worms were coiled, showed little movement, appeared swollen segmentally and occasionally had lost posterior segments. Changes from these initial responses occurred only after several hours when the worms either showed definite signs of recovery or began to disintegrate. As damage progressed the worms became swollen segmentally and appeared transparent with prominent and enlarged blood vessels. When the worms disintegrated they ruptured usually near the intersegmental septum, and cells, blood and body fluids were extruded. Any remaining tissue was a grey, opaque, motionless mass. In many cases complete dissolution occurred.

B. *Time course of mortality following oxygen exposure.* Worm survival data fell into one of three distinct patterns. Oxygen exposures of from one to eight hours resulted in negligible mortality throughout the two-week observation period. Mortality of these groups was indistinguishable from unexposed controls. Approximately half the worms died following exposures of nine to 12 hours; of these deaths virtually none occurred during the first six hours but approximately half occurred during the first 24 hours after exposure. Exposure times of 13 hours or more proved lethal to almost all worms during the first 120 hours. After these high doses approximately half the deaths occurred during the first six hours but many deaths were delayed beyond 24 hours. Representative survival curves are presented in Figure 1. Since a significant proportion of the deaths occur more than 48 hours after exposure, it is clear that observation times of greater than 48 hours must be used in the evaluation of factors influencing mortality of worms exposed to hyperbaric oxygen. When the data for all the worms within each of the high, intermediate and low survival groups and for the unexposed controls were pooled and

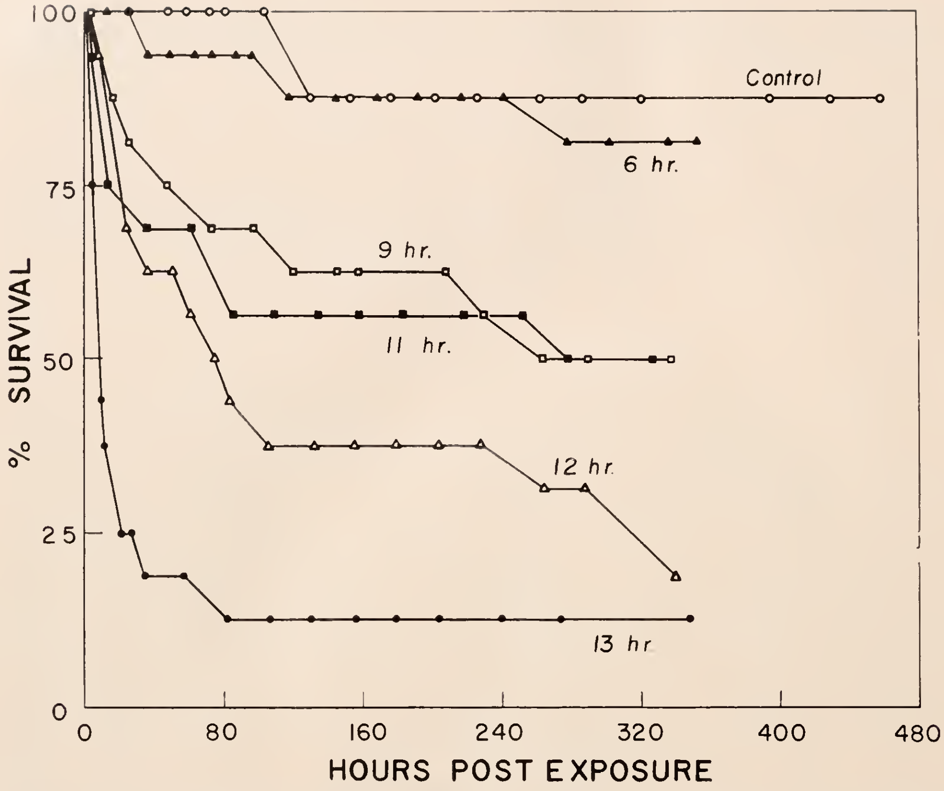


FIGURE 1. Post-exposure survival patterns of *T. tubifex* following low (1 to 8 hour), intermediate (9 to 12 hour) and high (13 to 15 hour) oxygen doses and survival pattern of unexposed controls.

hourly mortality rates calculated, high mortality rates within the first six to eight hours postexposure were found for both high and intermediate oxygen doses. In both the high dose group and the intermediate dose group a second peak of mortality occurred after a few days.

2. *Effect of pressure without increased oxygen*

Two experiments were run to distinguish the effects of pressure from those specifically related to oxygen. Two groups of worms cut for regeneration (24 worms per group) were exposed to three atmospheres of nitrogen added to one atmosphere of air. One group was exposed for 18 hours and one for 30 hours. Eight unexposed worms served as controls for each experiment. Worms removed from nitrogen were indistinguishable from unexposed controls after nitrogen treatment for as long as 30 hours. They showed neither the hyperactivity nor the contraction with lack of movement typical of oxygen-treated worms. Mortality of nitrogen-exposed worms after as long as 33 days was no greater than of unexposed controls. Results are presented in Table I.

3. *Interrupted exposure to hyperbaric oxygen*

The effect on survival of interrupting the oxygen exposure for various time periods was determined. A 16-hour exposure was chosen since this dose usually resulted in complete worm mortality but an exposure of half this duration was sublethal. Oxygen exposures of one half the lethal dose separated by various intervals could therefore be used to indicate the occurrence of repair processes. Worm groups were exposed to oxygen for eight hours, returned to atmospheric conditions and re-exposed to oxygen for a second eight-hour period.

The relationship between survival at 120 hours after oxygen exposure and the duration of interruption is presented in Figure 2. Interruption of the oxygen exposure clearly leads to an increase in worm survival indicating repair of some type of early damage. This repair process is one half maximal in four hours and maximal in approximately 16 hours. The time course of mortality and the survival at 120 hours for two half-lethal oxygen doses interrupted by four, six, eight, 12 and 20 hours closely resemble those resulting from single oxygen doses of from nine to 12 hours. A 16-hour interruption resulted in 100% survival.

TABLE I

Survival of T. tubifex *following exposure to three atmospheres nitrogen plus one atmosphere air (24 worms per nitrogen-exposed group)*

Duration of nitrogen exposure (hours)	Duration of experiment (hours)	Post-exposure survival (per cent)				
		24 hours	48 hours	168 hours	200 hours	Final
Experiment 1						
0	343	100.0	100.0	100.0	87.5	87.5
18	343	100.0	100.0	87.5	87.0*	83.3
Experiment 2						
0	792	100.0	100.0	82.0*	62.5	50.0
30	792	95.9	95.9	75.0*	62.0*	41.6

* By interpolation.

4. *Adaptation*

In view of the ability of *T. tubifex* to withstand alterations in environmental conditions, it seemed possible that these worms might adapt to conditions of increased oxygen tensions in their environment. This possibility was tested in the following manner. Worms were separated from the stock bucket, cut for regeneration and placed into five ml water in the paraffin blocks on the laboratory table under aluminum foil for 14 days. These worms were therefore more directly exposed to atmospheric oxygen concentrations without the protective mud environment available to them in the stock bucket or in nature. Before oxygen exposure these worms were recut for regeneration and exposed to an 18-hour oxygen dose along with worms freshly separated from the stock bucket and cut for regeneration. Results of two experiments are presented in Table II. Worms exposed to increased oxygen tensions in shallow depressions develop a significant resistance to

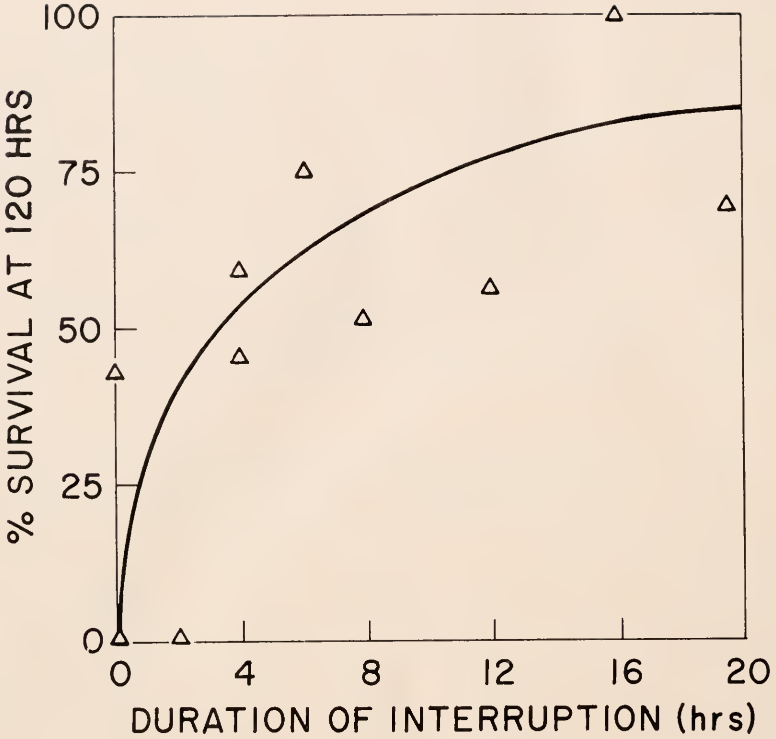


FIGURE 2. Per cent survival at 120 hours post exposure of *T. tubifex* following administration of two eight-hour oxygen doses interrupted by an interval in air.

hyperbaric oxygen while those exposed to oxygen immediately after separation from the stock bucket were markedly oxygen sensitive. This increased resistance to oxygen might represent true adaptation of the worms to conditions of higher oxygen tension or be the result of a conditioning of the environmental medium. To test these alternatives an exchange experiment was run. After 17 days of

TABLE II

Survival after 18-hour oxygen exposure of freshly-separated and of laboratory-adapted T. tubifex *(16 worms per group)*

Worm group	Post-exposure survival (per cent)			
	20 ± 1 hours	42 ± 1 hours	92 ± 3 hours	196 ± 12 hours
Experiment 1				
Freshly-separated	6.3	6.3	6.3	0.0
Adapted	100.0	100.0	93.8	93.8
Experiment 2				
Freshly-separated	43.8	31.3	6.3	6.3
Adapted	100.0	100.0	87.5	87.5

storage in paraffin blocks in the laboratory, worms were cut for regeneration. Some of these worms were replaced in the same depressions. Others were transferred into depressions containing fresh water. Freshly-separated and cut worms were placed either into fresh water or into the water from which the laboratory-stored worms had been removed. These four worm groups were simultaneously exposed to oxygen for 20 hours. Results are given in Table III.

As in the previous experiments, the worms kept in shallow depressions in the laboratory were markedly oxygen resistant. Transfer to fresh water may slightly decrease this resistance. There is no significant protection of freshly-separated worms by the conditioned water.

TABLE III

Survival of T. tubifex *after 20-hour oxygen exposure as affected by adaptation of worms and conditioning of medium (11 to 22 worms per group)*

Worm group	Post-exposure survival (per cent)			
	21 hours	47 hours	138 hours	229 hours
Freshly-separated, fresh water	0.0	0.0	0.0	0.0
Freshly-separated, conditioned water	9.0	9.0	—*	—*
Adapted, fresh water	68.2	68.2	59.1	59.1
Adapted, conditioned water	94.7	94.7	94.7	84.3

* No additional data.

DISCUSSION

The results presented here indicate that increasing duration of exposure to hyperbaric oxygen leads to both increased total mortality and increased rate of mortality of *T. tubifex*. Most published work on oxygen exposure of whole organisms has emphasized the immediate lethal effect of oxygen treatment. Immediate mortality after hyperbaric oxygen exposure has been reported for parasitic protozoa (Cleveland, 1925; Cleveland and Burke, 1956), *Paramecia* (Wittner, 1957; Gerschman, Gilbert and Frost, 1958a), *Drosophila* (Williams and Beecher, 1944), mice (Gerschman, Gilbert and Caccamise, 1958b), rats (Ozorio de Almeida, 1934; Stadie, Riggs and Haugaard, 1945), rabbits (Hederer and Andre, 1940) and dogs (Paine, Lynn and Keys, 1941). The present work demonstrates that the lethal effect of hyperbaric oxygen may not become manifest for several hours and that some lethally-exposed animals may survive for many days. Any proposed mechanism for oxygen poisoning may require a sequence of events before the final changes can be recognized.

Analysis of mortality rates indicates that two peaks of mortality occur following exposure to hyperbaric oxygen. This suggests that there may be two modes of death of *T. tubifex* following oxygen exposure or that within the worm population there are two types of individuals with different sensitivities to hyperbaric oxygen.

Since nitrogen at pressures equivalent to that of hyperbaric oxygen produced no significant mortality, the effects reported here can be attributed to oxygen and not to pressure itself.

Interruption midway through a 16-hour total exposure markedly increased the survival of *T. tubifex*. Interrupting oxygen exposures with an interval in air has also been reported to benefit dogs (Paine *et al.,* 1941), rats (Barach, Eckman, Oppenheimer, Rumsey and Soroka, 1944; Ackerman and Brinkley, 1966), guinea pigs (Penrod, 1956), mice (Wright, Weiss, Hiatt and Rustagi, 1966), rabbits (Ackerman and Brinkley, 1966) and *Drosophila* (Fenn, Henning and Philpott, 1967a; Fenn, Philpott, Meehan and Henning, 1967b). Recovery from eight-hour, half-lethal oxygen doses in *T. tubifex* is rapid being half maximal in four hours and maximal in approximately 16 hours. Since a 16-hour interruption led to 100% survival but a 20-hour interruption permitted only a 70% survival, it is not clear whether recovery goes to completion. Repair processes may be only partially complete. These findings appear to be similar to those reported by Fenn *et al.* (1967b) for *Drosophila.* It is possible that the kinetics of repair may be different for recovery from sublethal oxygen doses than for processes having the maximum effect. Incomplete recovery may be considered to be a residual injury. The interpretation is complicated by the occurrence of adaptation in this organism.

Adaptation to increased oxygen tensions has been reported previously. An increased tolerance to hyperbaric oxygen following an initial oxygen exposure (Smith, Heim, Thompson and Drinker, 1932) or after a gradual increase in the oxygen concentration extending over a number of days (Barach *et al.,* 1944) has been observed in the rat. The present report appears to be the first demonstration of adaptation to hyperbaric oxygen in an invertebrate.

Some invertebrates, *Daphnia* (Fox, 1955), *Chironomus* larvae (Fox, 1955) and *Artemia* (Gilchrist, 1954) respond to lowered environmental oxygen tensions with an increased synthesis of hemoglobin. The fact that *T. tubifex* does not synthesize additional hemoglobin under these conditions (Fox, 1955) lends support to Manwell's (1959) suggestion that hemoglobin in this organism may function as a buffer to protect against development of high oxygen concentrations in the tissues. It would be of interest to test for a correlation between the environmental oxygen concentration and the level of hemoglobin present in this organism.

I wish to express my deepest appreciation to Dr. Howard S. Ducoff for his advice and guidance throughout this study, to Dr. John D. Anderson for his continued interest and to the Department of Physiology and Biophysics for the use of their facilities.

SUMMARY

1. Exposure to hyperbaric oxygen caused *T. tubifex* to become hyperactive. After high doses of oxygen the worms became highly coiled and contracted and showed little movement. The behavioral response to oxygen exposure became complete only after several hours when the animals either disintegrated or began to resume normal behavior.

2. Exposure to four atmospheres absolute oxygen pressure for 13 to 15 hours caused high mortality of *T. tubifex*. Only an occasional worm survived. Exposure to four atmospheres absolute oxygen pressure for nine to 12 hours killed approximately half of the treated animals. Exposure to four atmospheres absolute

oxygen pressure for eight hours or less caused no greater mortality than in unexposed controls.

3. The lethal effects of oxygen exposure developed only after several hours. The lethal effect of a nine- to 12-hour exposure was half maximal in 24 hours and the effect of a 13- to 15-hour exposure in six hours. Some lethally-exposed worms survived for as long as 120 hours after 13- to 15-hour oxygen exposures.

4. Exposure to three atmospheres nitrogen added to one atmosphere of air for as long as 30 hours resulted in no more deaths than in unexposed controls and caused no change in worm behavior.

5. Interruption of a 16-hour oxygen exposure with return to atmospheric conditions enhanced survival of oxygen-treated *T. tubifex*. Increased duration of interruption led to increased survival of oxygen-treated worms. Recovery was half maximal in four hours and maximal in approximately 16 hours.

6. Adaptation to hyperbaric oxygen occurs in *T. tubifex* following several weeks of exposure to atmospheric oxygen tensions in shallow containers without a protective mud environment.

LITERATURE CITED

ACKERMAN, N. B., AND F. B. BRINKLEY, 1966. Cyclical intermittent hyperbaric oxygenation: A method for prolonging survival in hyperbaric oxygen, pp. 200–206. *In:* I. W. Brown, Jr. and B. G. Cox, Eds., *Proceedings of the Third International Conference on Hyperbaric Medicine.* National Academy of Sciences–National Research Council Publication No. 1404, Washington, D. C.

ALSTERBERG, G., 1922. Die respiratorischen Mechanismen der Tubificiden. *Lunds Universitets Årsskrift N. F. Avd. 2,* **18**: 1–175.

ANDERSON, J. C., 1956. Relation between metabolism and morphogenesis during regeneration in *Tubifex tubifex.* II. *Biol. Bull.,* **111**: 179–189.

BARACH, A. L., M. ECKMAN, E. T. OPPENHEIMER, C. RUMSEY, JR. AND M. SOROKA, 1944. Observations on methods of increasing resistance to oxygen poisoning and studies of accompanying physiological effects. *Amer. J. Physiol.* **142**: 462–475.

CHANCE, B., D. JAMIESON AND H. COLES, 1965. Energy-linked pyridine nucleotide reduction: Inhibitory effects of hyperbaric oxygen *in vitro* and *in vivo*. *Nature,* **206**: 257–263.

CHANCE, B., D. JAMIESON AND J. R. WILLIAMSON, 1966. Control of the oxidation-reduction state of reduced pyridine nucleotides *in vivo* and *in vitro* by hyperbaric oxygen, pp. 15–41. *In:* I. W. Brown, Jr. and B. G. Cox, Eds., *Proceedings of the Third International Conference on Hyperbaric Medicine.* National Academy of Science–National Research Council Publication No. 1404, Washington, D. C.

CLEVELAND, L. R., 1925. Toxicity of oxygen for protozoa *in vivo* and *in vitro:* Animals defaunated without injury. *Biol. Bull.,* **48**: 455–468.

CLEVELAND, L. R., AND A. W. BURKE, JR., 1956. Effects of temperature and tension on oxygen toxicity for the protozoa of *Cryptocercus. J. Protozool.,* **3**: 74–77.

FENN, W. O., M. HENNING AND M. PHILPOTT, 1967a. Oxygen poisoning in *Drosophila. J. Gen. Physiol.,* **50**: 1693–1707.

FENN, W. O., M. PHILPOTT, C. MEEHAN AND M. HENNING, 1967b. Recovery from oxygen poisoning in *Drosophila. Amer. J. Physiol.,* **213**: 663–670.

FOX, H. M., 1945. The oxygen affinities of certain invertebrate haemoglobins. *J. Exp. Biol.,* **21**: 161–165.

FOX, H. M., 1955. The effect of oxygen on the concentration of haem in invertebrates. *Proc. Roy. Soc. London, Series B,* **143**: 203–214.

GERSCHMAN, R., D. L. GILBERT AND J. N. FROST, 1958a. Sensitivity of *Paramecium caudatum* to high oxygen tensions and its modification by cobalt and manganese ions. *Amer. J. Physiol.,* **192**: 572–576.

GERSCHMAN, R., D. L. GILBERT AND D. CACCAMISE, 1958b. Effect of various substances on survival times of mice exposed to different high oxygen tensions. *Amer. J. Physiol.*, **192**: 563–571.

GILCHRIST, B. M., 1954. Haemoglobin in *Artemia*. *Proc. Roy. Soc. London, Series B*, **143**: 136–146.

HARNISCH, O., 1935. Versuch einer Analyse des Sauerstoffverbrauchs von *Tubifex tubifex* Müll. *Z. Vergl. Physiol.*, **22**: 450–465.

HEDERER, C., AND L. ANDRÉ, 1940. De l'intoxication par les hautes pressions d'oxygène. *Bull. Acad. Nat. Med.* (Paris), **123**: 294–307.

JAMIESON, D., AND B. CHANCE, 1966. Effect of high-pressure oxygen on the steady state of cytochromes in rat-liver mitochondria. *Biochem. J.*, **100**: 254–262.

JONES, J. R. E., 1938. Antagonism between two heavy metals in their toxic action on fresh-water animals. *Proc. Zool. Soc. London, Series A*, **108**: 481–499.

KOENEN, M., 1951. Vergleichende Untersuchungen zur Atmungsphysiologie von *Tubifex tubifex* M. und *Limnodrilus chaparèdeanus* R. *Z. Vergl. Physiol.*, **33**: 436–456.

MANWELL, C., 1959. Alkaline denaturation and oxygen equilibrium of annelid hemoglobins. *J. Cell. Comp. Physiol.*, **53**: 61–74.

OZORIO DE ALMEIDA, A., 1934. Recherches sur l'action toxique des hautes pressions d'oxygène. *C. R. Soc. Biol. Filiales*, **116**: 1225–1227.

PAINE, J. R., D. LYNN AND A. KEYS, 1941. Observations on the effects of the prolonged administration of high oxygen concentration to dogs. *J. Thoracic Surg.*, **11**: 151–168.

PALMER, M. F., 1968. Aspects of the respiratory physiology of *Tubifex tubifex* in relation to its ecology. *J. Zool. London*, **154**: 463–473.

PENROD, K. E., 1956. Effects of intermittent nitrogen exposures on tolerance to oxygen at high pressures. *Amer. J. Physiol.*, **186**: 149–151.

STADIE, W. C., B. C. RIGGS AND N. HAUGAARD, 1945. Oxygen poisoning III. The effect of high oxygen pressures upon the metabolism of brain. *J. Biol. Chem.*, **160**: 191–208.

SMITH, F. J. C., J. W. HEIM, R. M. THOMPSON AND C. K. DRINKER, 1932. Bodily changes and development of pulmonary resistance in rats living under compressed air conditions. *J. Exp. Med.*, **56**: 63–78.

WALKER, J. G., 1955. Effect of varied oxygen pressures upon the respiration of the annelid, *Tubifex tubifex*. *M.S. thesis University of Illinois,* Urbana, Illinois, 29 pp.

WILLIAMS, C. M., AND H. K. BEECHER, 1944. Sensitivity of *Drosophila* to poisoning by oxygen. *Amer. J. Physiol.*, **140**: 566–573.

WITTNER, M., 1957. Effects of temperature and pressure on oxygen poisoning of *Paramecium*. *J. Protozool.*, **4**: 20–23.

WRIGHT, R. A., H. S. WEISS, E. P. HIATT AND J. S. RUSTAGI, 1966. Risk of mortality in interrupted exposure to 100% O_2: role of air vs. lowered P_{O_2}. *Amer. J. Physiol.*, **210**: 1015–1020.

Vol. 138, No. 3 June, 1970

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

JET PROPULSION OF SQUID[1]

K. S. COLE AND D. L. GILBERT

Laboratory of Biophysics, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20014

A detailed description and measurements of jet propulsion in *Loligo* has been published recently by Packard (1969). He gives results obtained from newly-hatched, juvenile, and adult squid (*Loligo vulgaris*) by the use of strobe- and cine-records—presumably made in the aquarium at Naples. His results provide a welcome confirmation of an older unpublished observation and a reminder of

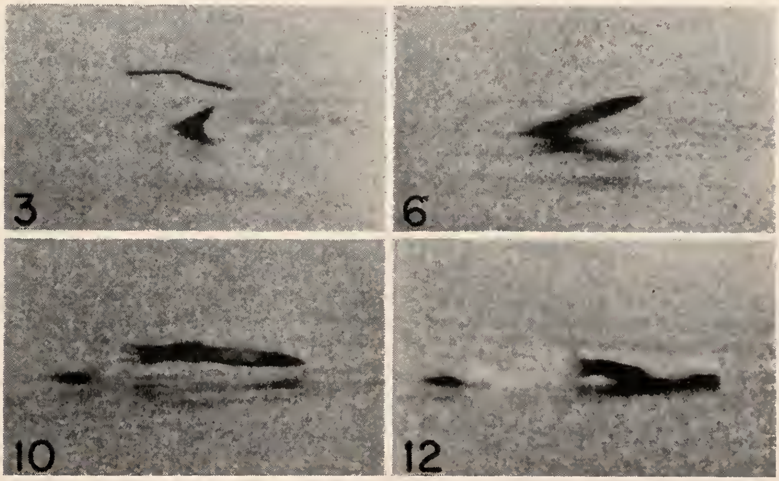


FIGURE 1. Photographs of a squid (*Dosidicus gigas*) jumping out of water. Numbers refer to frame numbers. Time between each consecutive frame number was $\frac{1}{16}$ second. Note that this is a backwards leap.

---

[1] It has long been the policy of THE BIOLOGICAL BULLETIN not to accept very short papers or brief notes. Because of the distinguished contributions of Dr. Kenneth S. Cole to the Marine Biological Laboratory, an exception is being made in this case. This is only the second such paper in the last twenty volumes of the BULLETIN—*Editor.*

Library of Congress Card No. A38-518
Copyright © 1970, by the Marine Biological Laboratory

another made in open water. We now report these two observations as a footnote to Packard's paper.

In the summer of 1936, H. J. Curtis and K. S. C. saw *L. pealei* feeding on "shiners" near a shore of Long Island Sound. As they attacked from below, some squid broke the surface and a few cleared it by an appreciable fraction of their length. Assuming a length of 25 cm, it was guessed that the center of gravity rose 20 cm (h). From $v^2 = 2gh$ the initial velocity (v) was 200 cm/sec. The corresponding figure of 4 knots has often been used as an illustration of the squid jet power and now the agreement with the maximum found for *L. vulgaris* (Packard, 1969) shows that guesses can be lucky.

During work at Montemar, Chile, in 1964, D. L. G. made a movie (Fig. 1) from a small boat of *Dosidicus gigas* in a leap above water. Guessing a mantle length of 4 feet it was no great surprise to get an emerging speed of about 3.5 knots. However, this figure increased to a maximum velocity of almost 14 knots between frames 8 and 9, Figure 2. Frame 12 of Figure 1 and the graph of Figure 2 suggest that a continuing ejection of water produced this acceleration. This example provides a striking illustration of amphibious jet propulsion.

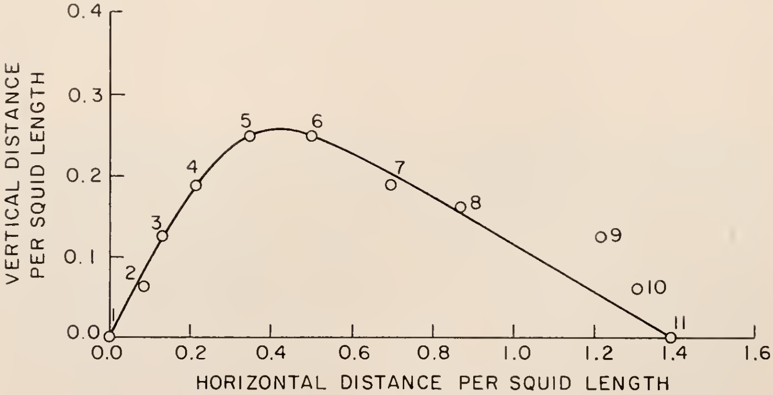


FIGURE 2. Plot of the caudal end of the squid (in Figure 1) in air with respect to the point of origin of the emergence from the water surface, as estimated from the film record.

LITERATURE CITED

PACKARD, A., 1969. Jet propulsion and the giant fibre response of *Loligo*. *Nature*, 221: 875–877.

Reference: *Biol. Bull.*, **138**: 247–261. (June, 1970)

OSMOTIC AND IONIC REGULATION IN *LUMBRICUS TERRESTRIS* L.[1]

THOMAS H. DIETZ[2] AND RONALD H. ALVARADO

Department of Zoology, Oregon State University, Corvallis, Oregon 97331

Earthworms normally live in moist soil but during rainy seasons they are exposed to an aquatic environment. In the laboratory they survive several months in freshwater of the appropriate ionic composition (Roots, 1956). Because of the difficulties in controlling soil conditions it is of advantage to study the basic features of osmotic regulation under freshwater conditions. This is particularly important in the analysis of ionic regulation where rigorous control of the external bath is essential. In fact most of the published information on hydromineral metabolism in *Lumbricus* is on animals in freshwater (Adolph, 1927; Maluf, 1939, 1940; Wolf, 1940; Bahl, 1945; Ramsay, 1949ab; Kamemoto, Spaulding and Keister, 1962; van Brink and Rietsema, 1949). While these studies have established many important features of osmotic regulation in *Lumbricus,* the mechanisms of osmotic and, particularly, ionic regulation in these animals are poorly understood.

For this reason we undertook a systematic analysis of hydromineral metabolism in the earthworm living under freshwater conditions. Secondly, we analyzed some of the adjustments in hydromineral balance these animals undergo when transferred from soil to freshwater. Finally we sought to obtain some preliminary information on the control of the various mechanisms involved in osmotic and ionic regulation in these animals.

METHODS

1. *Animals*

Lumbricus terrestris weighing 1 to 10 g (mean 3 g) were obtained from a local bait dealer and kept in a leaf mulch soil at 14 ± 1° C, on a 14 hr photoperiod. Water was added to the soil periodically but soil moisture was not rigorously controlled. Most animals were equilibrated in artificial pond water (0.5 m*M* NaCl, 0.05 m*M* KCl, 0.40 m*M* $CaCl_2$ and 0.20 m*M* $NaHCO_3$) for at least one week prior to use. The bath was continuously aerated.

2. *Coelom, crop and rectal fluid*

Coelomic fluid (CF) was collected by inserting a small, pointed capillary tube through the body wall into the coelom of worms immobilized by packing in ice.

---

[1] This investigation was supported by grants from the National Institutes of Health (5-F1-GM-35,422 to T. H. Dietz and AM-10508 to R. H. Alvarado). Parts of this work were submitted by T. H. Dietz to the Graduate School of Oregon State University in partial fulfillment of the requirements for the Ph.D. degree.

[2] The present address of T. H. Dietz is: Cardiovascular Research Institute, School of Medicine, University of California, San Francisco, California, 94122.

The crop was exposed by cutting through the skin. Adherent CF was removed by blotting and a pointed capillary tube inserted into the crop and the fluid collected. Rectal fluid was collected by inserting a capillary tube through the anus. Particulate matter was removed by centrifugation.

3. *Chemical analyses*

Sodium and potassium concentrations in diluted CF, rectal and crop fluid were determined by flame photometry; chloride by electrometric titration. Ammonium was determined colorimetrically by direct nesslerization. Total solute was determined on undiluted samples using a Mechrolab vapor pressure osmometer.

Total Na^+, K^+ and Cl^- contents of earthworms were also determined. The animals were dried to a constant weight at 105° C. For Na^+ and K^+ determination the carcasses were digested with hot (40–50° C) concentrated HNO_3. The digest was diluted for analysis by flame photometry. For Cl^-, the dried animals were ground to a powder and extracted with distilled water for 12 to 24 hr at 3° C. Particulate matter was removed by centrifugation or filtration and the water extract was analyzed for Cl^- by electrometric titration.

4. *Coelomic fluid clearance*

Clearance from the CF of C-14-inulin, high molecular weight dextran, and glycine was measured on separate animals. The labeled compound diluted in an iso-osmotic saline solution was injected into the coelomic cavity; each animal was placed in pond water (PW) and left one hour to allow distribution of the isotope in the CF (separate experiments indicated distribution was complete within two hours). Each animal was then rinsed, weighed and transferred to 10 ml of PW. The containers with the animals were gently shaken at 15 ± 1° C. Samples were removed from the bath at one hour intervals for 4 hr and assayed for radioactivity by liquid scintillation methods. At the end of the experiment a CF sample was collected and counted. The rate of counts appearing in the bath is the product of the urine radioactivity and the volume excreted. From the bath and CF radioactivity the clearance of each substance was determined. Since the total radioactivity loss from the CF was less than 10 per cent, the CF was considered constant. The technique assumes that drinking is negligible in these animals (see below).

5. *Ion fluxes*

Sodium and chloride influx was measured with Na-22 or Cl-36, respectively. The radioactive isotope was added to a bath of known volume and ionic composition. Quantitative samples were withdrawn from the bath at specified intervals, placed in a planchet, evaporated to dryness, and assayed for radioactivity. A separate sample was assayed for Na^+ by flame photometry or Cl^- by electrometric titration. The influx was calculated from the rate of diminution of radioactivity in the bath (see Jørgensen, Levi and Ussing, 1946). The net flux was obtained from the chemical analysis. The efflux then was computed by difference (net flux = influx − efflux).

6. *Transepithelial electrical potentials*

The transepithelial electrical potential (TEP) generated by earthworms *in vivo* was measured as described by Dietz, Kirschner and Porter (1967). The basic bathing solution was 1 m*M* K_2SO_4 to obtain a conducting medium. Solutions were added to the bath to give the required ionic composition and concentration.

7. *Statistical treatment*

All values for populations of animals are presented as the mean ± one standard error of the mean. Differences between means were analyzed by the student "t" test and considered significant if $P < 0.05$.

RESULTS

1. *Effect of ionic composition of the bath on CF composition*

Groups of six to eight worms were kept in baths of varying concentration of NaCl for 10 to 13 days whereupon the ionic composition of their CF was determined. The results are shown in Figure 1. Earthworms hyper-regulate with respect to Na^+ and total solute in solutions below 78 m*M* NaCl. Above this the CF remains slightly hyper-osmotic but the Na^+ concentration is equal to the bath. Chloride is hyper-regulated in solutions below 40 m*M* NaCl but hypo-regulated above this. Over the entire range, Na^+ and Cl^- account for most of the solute in CF. There is a slight increase in the total solute above that contributed by Na^+ and Cl^- in the animals acclimated to 126 and 150 m*M* NaCl. The source is unknown. Potassium concentration is about 3 m*M*/l and remained constant.

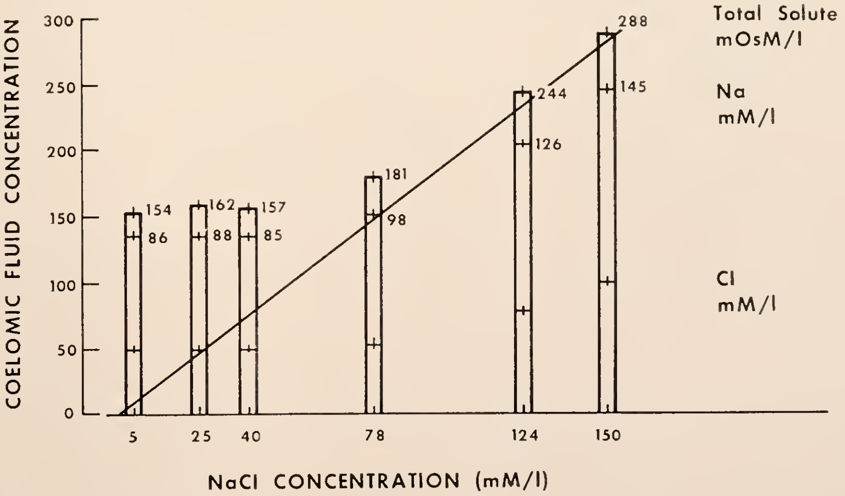


FIGURE 1. Coelomic fluid composition of *Lumbricus terrestris* acclimated 10 to 13 days in different NaCl solutions. Sodium is added to chloride in each column. The number adjacent to each bar is the mean concentration of the ion indicated (N = 8). Vertical lines indicate ± one S.E. The diagonal line represents iso-osmoticity.

Although survival was high in solutions containing at least 5 mM NaCl we found that earthworms do not survive more than two weeks in dilute NaCl solutions (< 1.0 mM) or distilled water. Yet they survived indefinitely in an artificial pond water containing a dilute mixture of ions.

2. *Adjustment to PW*

Upon transfer from soil to PW earthworms gain weight for about two days as shown in Figure 2. A steady state is then reached at a weight of 115% of the weight in soil (B_0). This increase in weight reflects the net accumulation of water which initially cases a dilution of the solute in the CF. The reduction is transitory, however, because after seven days in PW concentrations of ions in the CF are very similar to those of soil animals (Table I) and this does not involve a loss of water (Fig. 2). Either net quantities of ions are accumulated from the bath or ions are mobilized from stores within the animal. Evidence for the former hypothesis is shown in Figure 2. Animals were transferred individually from soil to a limited volume of PW. Changes in bath concentrations of Na^+, Cl^- and K^+ were followed and the net exchanges calculated. A net uptake of Na^+ and Cl^- which begins almost immediately is apparent. In the experiment depicted, the animals came into a steady state with respect to Cl^- in three days but continued to accumulate Na^+ throughout the experiment. In other experiments animals did approach a Na-steady state in seven days. It is particularly noteworthy that the uptake of Cl^- does

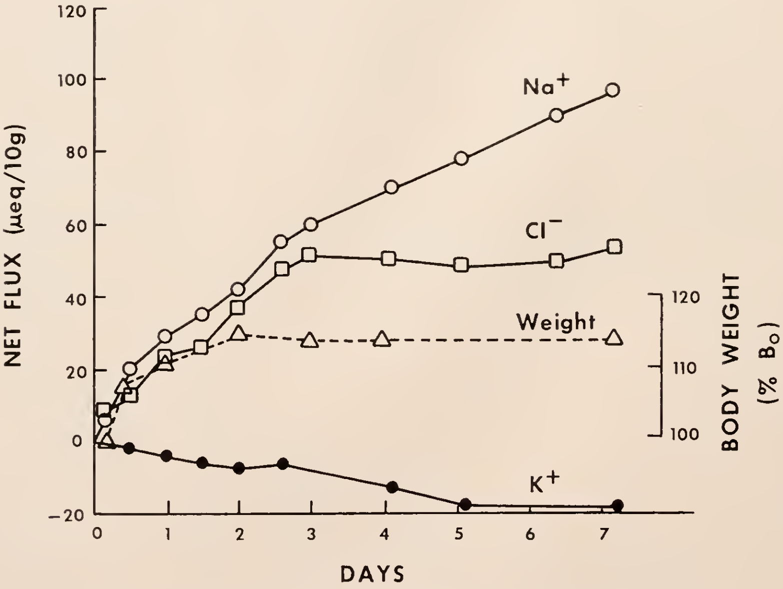


FIGURE 2. Net exchange of ions and water for worms transferred from soil to 50 ml of PW. Each point is based on the mean of eight worms. The initial body weight was 2.7 ± 0.4 g.

TABLE I

Ionic composition and concentrations of ions in coelomic fluid (CF) of Lumbricus *acclimated to soil or pond water*

Measurement	Units	Soil	Pond water
Water content	ml/10 g†	8.4 ± 0.1 (18)	8.8 ± 0.0* (22)
Total Na^+	μeq/10 g	235 ± 3 (8)	373 ± 15* (12)
Total K^+	μeq/10 g	345 ± 15 (8)	365 ± 15 (12)
Total Cl^-	μeq/10 g	172 ± 8 (8)	174 ± 13 (12)
CF Na^+	meq/l	71 ± 2 (14)	75 ± 1 (24)
CF K^+	meq/l	4 ± 0 (14)	3 ± 0 (24)
CF Cl^-	meq/l	48 ± 1 (14)	47 ± 1 (24)
CF Total solute	mOsm/l	154 ± 2 (10)	159 ± 2 (15)

† Wet weight.
* Significantly different from soil animals ($P < 0.05$).

not equal that of Na^+, and the time courses of uptake are not necessarily similar. Potassium is continually lost.

These data show that earthworms have a delicately controlled iono-regulatory system. Presumably dilution of the body fluids upon placement in PW activates the iono-regulatory systems. The nature of this activation will be explored in a subsequent section (control of ionic regulation). However, first we shall examine water and ion exchanges in animals acclimated to PW.

3. *Water exchanges*

Earthworms in PW can take up water across the gastro-intestinal tract and/or the skin; they can lose water by nephridial excretion or rectal excretion. In PW, worms open their mouths periodically and they will also feed on particulate matter in the water. To determine if fluid is ingested, worms were placed in PW containing C-14-inulin (0.1 μc) and left for nine days, whereupon samples of the crop, midgut and CF were collected and assayed for radioactivity.

The crop fluid radioactivity ranged between 29 and 69% of the bath radioactivity. The midgut fluid was between 39 and 69% of the bath activity but the CF did not exceed 5% of the bath activity. Apparently these animals drink. The drinking rate was estimated from a water extract of dried worms which had been in PW with C-14-inulin for 10 hr. The mean rate for five animals was 4.2 ± 1.0 μl/10 g-hr.

Measurement of the rate of urine flow is difficult, but a maximal estimate can be obtained by measuring clearance rates of certain substances from the CF. The material must not be stored or metabolized, its entry into the nephridia must be unrestricted and there can be no reabsorption from the nephridia. If the presumptive urine entering the nephridia is coelomic fluid entering via the nephrostome, then all substances satisfying the assumptions should give the same clearance value. We checked C-14 labeled glycine, inulin and a high molecular weight dextran expecting that the first would be reabsorbed while the last two would meet the above mentioned requirements. Table II shows that PW-acclimated worms clear inulin and dextran at similar rates and glycine clearance is only about $\frac{1}{7}$ as great.

TABLE II

The rates of clearance of inulin, dextran and glycine from the coelomic fluid of the earthworm, Lumbricus terrestris, *immersed in pond water*

Substance	Clearance (μl/10 g-hr)
PW acclimated	
Inulin	70 ± 11 (7)
Dextran	76 ± 19 (5)
Glycine	10 ± 4 (5)
Soil acclimated	
Inulin	10 ± 2 (4)
Inulin	83 ± 19 (6)

Soil-acclimated animals, placed in PW, responded variably. Six out of ten animals had similar clearance rates comparable to PW animals while in four the rates were only ⅛ as fast. The reason for this difference is not known but it suggests that animals in soil have a low clearance rate which is elevated in response to exposure to PW. Individuals may differ in the time required to elicit the response even though water is entering osmotically.

Curiously, the clearance of inulin and dextran remained unchanged when PW-acclimated worms were placed in iso-osmotic sucrose solution for six hr. Apparently the rate of osmotic uptake of water does not immediately affect the rate of clearance of CF.

No urine analyses were performed so the amount of water absorbed by the nephridia could not be determined. It is likely that some reabsorption occurs so that the rate of urine flow would be less than the clearance rate of inulin (75 μl/10 g-hr).

Earthworms in PW void a fluid from the anus. We have not measured the rate of elimination of this rectal fluid but Wolf (1940) estimated a rate of 22 μl/10 g-hr. Animals injected (CF) with C-14-inulin or C-14-dextran did not void either compound through the anus. The source of the rectal fluid is not known.

4. *Sodium and chloride balance*

Worms acclimated to PW maintain a steady state with respect to Na^+ and Cl^-. The unidirectional fluxes are given in Table III. Chloride influx is greater than Na^+ influx possibly because $[Cl^-]$ is greater than $[Na^+]$ in PW (see below).

Solutes may be lost through the gastro-intestinal tract, the nephridia, or across the skin. Crop fluid is remarkably similar to CF in $[Na^+]$, $[K^+]$, and total solute, but considerably lower in $[Cl^-]$ (Table IV). Since drinking is minimal in PW, most of the crop fluid must be derived from the body fluids. We never observed regurgitation of fluid through the mouth so that the crop fluid must pass posteriorly through the intestine and be subjected to alteration. Rectal fluid is more dilute than CF or crop fluid (Tables II and IV) and approaches published values for earthworm urine (Bahl, 1945; Ramsay, 1949 ab). The mechanism of formation

TABLE III

Mean Na^+ and Cl^--unidirectional fluxes in PW-acclimated Lumbricus terrestris.
The average weight of the worms was 4.4 ± 0.3 g.

Ion	N	Flux (μeq/10 g-hr)	
		M_i	M_0
Na^+	15	0.32 ± 0.04	0.37 ± 0.07
Cl^-	15	0.88 ± 0.10	0.90 ± 0.14

of crop fluid and its subsequent alteration remain unknown. It would seem that the possibility of a filtration-reabsorption system comparable to that found in excretory organs in other fresh-water animals might be worth exploring (see discussion). If Wolf's (1940) estimate of the rate of rectal fluid elimination (0.022 ml/10 g-hr) is correct than about 36% of the Na^+ and 32% of the total Cl^- is lost via this route. The rest must be lost through the nephridia or the skin.

We have not been able to obtain a direct estimate of the nephridial component of the efflux. However, it is possible to establish some limits. The clearance of inulin (75 μl/10 g-hr) gives a maximal estimate of the rate of urine flow. The urine concentration of Na^+ and Cl^- was not measured but if there were no loss of these ions across the skin the urine concentration could not exceed 3.2 μeq/ml for Na^+ and 8.1 μeq/ml for Cl^-. Published values for urine $[Cl^-]$ are about 3.5 μeq/ml (Bahl, 1945; Ramsay, 1949ab) so that a maximum of 0.26 μeq Cl/10 g-hr, or 29% of the Cl^- efflux, would be through the nephridia. This means that at least 0.35 μeq/10 g-hr or 39% of the Cl efflux is across the skin. No information is available on urine Na^+ concentration.

To demonstrate active transport of an ion, it must be shown that the ion is accumulated against an electrochemical gradient. Transepithelial electrical potentials (TEP) were measured on worms acclimated to PW and exposed to a range of NaCl or Na_2SO_4 concentrations. The TEP is a logarithmic function of the Na^+ concentration and independent of the anion. At low $[Na^+]$ (0.2 meq/l) the CF is negative to the bath (−20 to −40 mV). When the bath $[Na^+]$ is greater than 20 m*M* the inside became positive. The TEP values of worms acclimated to PW and measured in PW were between −30 and −12 mV.

TABLE IV

Ionic composition of crop fluid and rectal fluid of Lumbricus *acclimated to pond water*

Ion	Concentration (m-Moles/l)	
	Crop	Rectal fluid
Na^+	62 ± 3 (14)	6 ± 2 (7)
K^+	4 ± 0 (10)	3 ± 0 (3)
Cl^-	2 ± 0 (14)	13 ± 5 (9)
N	—	14 ± 2 (7)
Total solute*	153 ± 4 (16)	36 ± 10 (6)

* mOsm/l.

If the inward and outward permeability coefficients for an ion are the same, then the passive diffusion of that ion should obey the flux-ratio equation (Ussing, 1949):

$$M_i/M_o = (c_o/c_i)\exp(zFE/RT) \tag{1}$$

where the subscripts i and o refer to the inside and outside compartments, M is the flux in μeq/cm^2-hr; c is the ionic concentration in μeq/cm^3; z is the valence of the ion; F is the Faraday, 96,500 joules/volt mole; E is the potential difference across the membrane in volts; R is the gas constant 8.3 joule/degree mole and T is the absolute temperature. In a steady state the flux ratio (M_i/M_o) is equal to one. If an ion is passively distributed then a calculation of the expected flux ratio should equal the observed flux ratio. For animals in PW the calculated flux ratios would be 0.004 and 0.059 for Na^+ and Cl^-, respectively, assuming that the TEP is 20 mV (inside negative). Transport of both Na^+ and Cl^- is thermodynamically active.

The integument is the primary site of Na^+ transport. This was determined by measuring the influx of Na-22 on seven worms which had their anterior and posterior ends ligated with heavy cotton string. The influx over the 10 hr interval was 1.76 ± 0.16 μeq Na^+/10 g-hr with a net uptake of 0.12 ± 0.12 μeq Na^+/10 g-hr. Handling stress is probably responsible for the high influx.

To further characterize the Na^+ transport mechanism, experiments were performed measuring the influx of Na^+ over a range of external Na^+ concentrations. The basic solution for these studies was PW with different amounts of NaCl added or deleted. Worms were acclimated to PW prior to the flux measurements shown in Figure 3. The rate of Na^+ transport is dependent on bath [Na^+] at low concen-

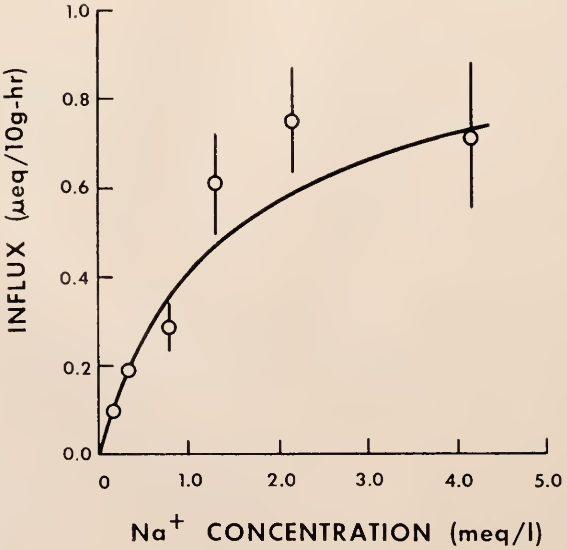


FIGURE 3. The effect of external Na^+ concentration on the influx of Na^+ into PW-acclimated worms. Each point represents a mean of at least six animals. Vertical lines represent ± one S.E. The mean weight of the animals was 4.3 ± 0.2 g (56).

trations and Michaelis-Menten kinetics apply. The maximum influx (V_{max}) for PW-acclimated worms is 0.96 μeq/10 g-hr. The Na^+ concentration at which the influx is half V_{max}, or K_s, is 1.3 mM Na/1.

5. *Control of* Na^+ *and* Cl^- *balance*

Experiments described in a previous section show that the dilution of the body fluids following transfer of worms from soil to PW is due to a net uptake and retention of water. Eventually Na^+ and Cl^- were accumulated restoring their CF concentrations to levels comparable to soil animals. The kinetics of this adjustment were analyzed by following Na^+ and Cl^- fluxes during various parts of the adjustment period. The results are shown in Table V.

TABLE V

Na^+ and Cl^- unidirectional fluxes in worms transferred from soil to PW. The animals weighed 3.4 ± 0.1 g (48). Each value represents a mean of 8 animals

Hours in PW	Flux (μeq/10 g-hr)			
	Na^+		Cl^-	
	M_i	M_0	M_i	M_0
0–24	1.34 ± 0.15	0.21 ± 0.05	2.94 ± 0.23	1.06 ± 0.14
36–72	0.84 ± 0.09	0.51 ± 0.06	1.87 ± 0.24	1.60 ± 0.20
90–130	0.52 ± 0.14	0.51 ± 0.14	0.73 ± 0.15	0.86 ± 0.11

The net uptake of Na^+ and Cl^- is most pronounced during the first interval although some is accumulated during the second. By the third interval a steady state is restored. The net uptake results primarily from an elevated influx of each ion, although in the case of Na^+ there is also a reduction in the efflux during the first period.

When worms enter PW there is both an increase in water content and a dilution of the body fluids either one of which could serve as the stimulus for the responses indicated above. By exposing PW-acclimated worms to distilled water (DW), it is possible to reduce the solute concentration of the CF without affecting water content. *Lumbricus* will not tolerate prolonged exposure to DW (50% mortality in one week), however, they will survive three or four days. During this period they suffer a net loss of Na^+ which is highest during the first four hr (0.35 to 2.39 μeq Na^+/10 g-hr) and then stabilizes to a mean value of 0.28 ± 0.05 μeq/10 g-hr between 4 and 72 hr. Even though the bath volume was limited (50 ml) these worms did not come into a steady state with respect to Na^+. We did observe that Na^+ loss in the presence of Ca^{++} in the bath is 30% lower than the loss to distilled water.

The loss of ions experienced during exposure to distilled water is reflected by a reduction in their concentration in the CF. Thus after three days [Na^+] was reduced 19% and [Cl^-] 39% relative to PW-acclimated animals. The animals do not gain weight in DW.

When returned to PW, salt-depleted worms experience a net uptake of Na^+ and Cl^- which can be attributed primarily to a marked increase in the influx of each ion (Table VI). It appears that the reduction in CF ion concentration stimulates the transport systems.

TABLE VI

Mean Na^+ and Cl^- unidirectional fluxes for salt-depleted worms placed in PW. Each value represents an average of 4 animals measured over 22 hours. The animals weighed 2.9 ± 0.3 g

Ion	N	Flux (μeq/10 g-hr)	
		M_i	M_0
Na^+	4	2.83 ± 1.01	1.35 ± 0.46
Cl^-	4	3.88 ± 0.86	0.78 ± 0.68

6. *Independence of Na^+ and Cl^- transporting systems*

Since salt depletion stimulates both Na^+ and Cl^- transport systems an experiment was performed to determine if there is a dependency between them. Six worms, salt depleted for four days, were placed in individual containers having either 0.35 m*M* Na_2SO_4 or 1.30 m*M* KCl and the Na^+ and Cl^- flux, respectively, was measured over 21 hr (Table VII). Clearly sodium and chloride can be transported independently although at a lower rate than that measured from PW.

TABLE VII

*Mean Na^+ and Cl^- unidirectional fluxes for salt-depleted worms exposed to 0.3 m*M* Na_2SO_4 or 1.25 m*M* KCl. Each value represents an average of 3 animals measured over 21 hours. The animals weighed 4.6 ± 0.7 g*

Ion	N	Flux (μeq/10 g-hr)	
		M_i	M_0
Na^+	3	0.58 ± 0.05	0.36 ± 0.01
Cl^-	3	1.69 ± 0.60	0.67 ± 0.32

Potassium is not accumulated and separate studies revealed that $^{35}SO_4^=$ is not taken up from the bath. In order to preserve electroneutraility the net uptake of Na^+ from Na_2SO_4 and of Cl^- from KCl must involve exchange with an endogenous ion of like charge. Either NH_4^+ or H^+ could be exchanged for Na^+ and HCO_3^- or OH^+ for Cl^-. All of these are metabolic products which are produced at rates commensurate with such an exchange system.

DISCUSSION

Survival of earthworms in an aquatic medium is dependent on the ionic composition of the bath. They do not survive more than one or two weeks in distilled water or 1.0 m*M* NaCl. They will survive in more concentrated solutions of

NaCl (5 m*M* or greater) or in an artificial pond water. The basis for these observations has not been determined. Calcium probably has a direct effect on permeability of exposed epithelia. Sodium loss to dilute $CaCl_2$ solutions is slower than to distilled water. A similar observation has been made on the ammocoetes larva by Morris and Bull (1968). Most of our experiments were performed on animals in an artificial pond water containing Na^+, K^+, Ca^{++}, HCO_3^- and Cl^- (see above). We have kept worms several months in this medium. The ionic concentrations in PW are similar to soil water (Davson, 1964).

Animals acclimated several days in PW maintain a constant body weight despite a large osmotic gradient favoring the uptake of water. The mechanisms for maintaining water balance are in most respects similar to those of other fresh-water animals (Krogh, 1939; Potts and Parry, 1964). These include a relatively low permeability to water, low drinking rate and the capacity to excrete a urine hypoosmotic to the body fluids. In addition the gastro-intestinal tract of earthworms may be involved in the elimination of water.

The rate of urine production was not estimated directly, but if our assumptions relating to the clearance of inulin and high molecular weight dextran are valid, the rate of urine production cannot exceed the rate of clearance of these compounds, or about 75 μl/10 g-hr. The rate of excretion of rectal fluid has been estimated by Wolf (1940) at 22 μl/10 g-hr. Thus the rate of elimination of water is less than 100 μl/10 g-hr (24% of body weight/day).

In the steady state this value equals the osmotic uptake of water and is comparable to what is found in a variety of fresh-water animals (Krogh, 1939, Potts and Parry, 1964). This is lower than the value of 250 μl/10 g-hr estimated by Wolf (1940). The discrepancy can probably be attributed to differences in technique since he blotted the animals and this may have increased their permeability to water.

There are two sites of entry of water, the integument and the gut. Maluf (1939) has shown that ligation of anterior and posterior ends of worms does not prevent the net uptake of water when worms are transferred from soil to water. Earthworms are like most fresh-water animals in that drinking is minimal. By placing animals in a bath containing C-14-inulin we have found that the volume of bath passing into the digestive tract is about 4 μl/10 g-hr. If all of this were absorbed it would account for about 4% of the water taken up by the worms. Obviously the major route of uptake of water is across the body surface. We have no accurate data of the area of a 10 g worm but 50 cm^2 would seem reasonable (surface area = 10 $Wt^{2/3}$). The permeability constant for water then would be about 2 μ/sec. This is slightly higher than most fresh-water animals which usually range between 1 and 0.1 μ/sec (Potts and Parry, 1964), but comparable to others such as the frog (4 μ/sec).

The formation of urine by nephridia involves movement of CF through the nephrostome by action of cilia. This presumptive urine is then modified by reabsorption and possibly secretion. Boroffka (1965) has shown that dilution of urine occurs primarily in the distal portion of the nephridia by active reabsorption of Na^+. The tubule must be relatively impermeable to water.

Rectal fluid is also quite dilute whereas crop fluid is virtually iso-osmotic with CF. The origin of crop fluid remains unknown however conceivably it is a filtrate of blood. Blood is quite similar to CF (Kamemoto *et al.,* 1962). Thus blood entering the posterior esophagus (region of the calciferous glands) could be filtered into the G-I tract. The presence of large blood sinuses separated from the lumen of the esophagus by a thin layer of cells is structurally suited for filtration (Semal, 1959; Laverack, 1963). We were unable to demonstrate the filtration of inulin into the gut. Perhaps the molecule is too large. The [Cl^-] of crop fluid is very low (Table IV) probably because there is an exchange of Cl^- for HCO_3^- between blood and the gut. In the presence of Ca^{++} some of the HCO_3^- would form $CaCO_3$ (Robertson, 1936).

As the fluid passes posteriorly solute could be reabsorbed to form the hypo-osmotic rectal fluid. Some of the Na^+ removed from the gut may be in exchange for NH_3 (Table IV; Tillinghast, 1967). This hypothesis is highly speculative but would appear to be a more satisfactory explanation than an alternative which would be transport of water from blood or CF to the gut to produce a hypo-osmotic gut fluid.

Living in PW without food, earthworms hyper-regulate Na^+, Cl^-, K^+, and total solute. The turnover time of Na^+ and Cl^- would be 49 and 8 days, respectively. Ions are lost across the body surface by diffusion and excretion through the gut and urine. For Cl^- the approximate contribution of each of these would be: rectal fluid 32%, urine 29%, and skin 39%. We do not have an accurate measure of urine Na^+ so that we cannot partition the Na^+ efflux. It is noteworthy that urine Na^+ must be less than 3 μeq/ml, assuming a rate of urine production of 75 μl/10 g-hr and rectal fluid excretion of 22 μl/10 g-hr and the concentrations indicated in Tables I and IV.

In PW earthworms remain in a steady state with respect to Na^+ and Cl^- because they can transport each ion from the bath to the body fluids against an electrochemical gradient. For Na^+, at least, this capacity resides primarily in the integument since animals with their mouth and anus blocked have an influx of Na^+ equal to or greater than normal animals. We have not studied Cl^- transport in this respect.

The influx of Na^+ is a function of external [Na^+] at lower concentrations (< 3 mM Na/l) but at higher concentrations appears to be Na-independent (Fig. 3). Since the diffusive component of the influx is negligible over this concentration range, the magnitude of the influx suggests that the membrane permeability to Na^+ is low. The curve relating M_i to external [Na^+] approximates a rectangular hyperbola which can be expressed by an equation analogous to the Michaelis-Menten equation for enzyme kinetics where V corresponds to influx:

$$V = \frac{V_{\max}[\mathrm{Na}^+]}{K_s + [\mathrm{Na}^+]} \qquad (2)$$

For the earthworm adapted to PW and tested at various concentrations of Na^+, $V_m = 1$ μeq/10 g-hr and $K_s = 1.3$ mM/1. The V_m value is comparable to values of some other fresh-water animals, for example the crayfish, 1.5 (Shaw, 1963), and the salamander, 3.0 (Alvarado and Dietz, 1970). The K_s of earthworms is higher than for most freshwater invertebrates but lower than marine forms. For example K_s for the fresh-water crayfish *Astacus* is 0.2 mM Na^+/l whereas for *Carcinus*, a brackish-water crab, it is 20 mM Na^+/l (Potts and Parry, 1964).

Compared to brackish water and marine annelids, earthworms exchange Na^+ and Cl^- at a much lower rate. For example *Nereis* in dilute sea water (9%) exchanges Na^+ at about 80 μeq/10 g-hr (12° C) and Cl^- at about 160 μeq/10 g-hr (24° C) (Fretter, 1955; Jørgensen and Dales, 1957). Even when transferred to freshwater, *Nereis* has an influx of Cl^- of 55 μeq/10 g-hr (Jørgensen and Dales, 1957). Adaptation to freshwater in this Phylum has involved a marked reduction in the rate of exchange of ions between the animals and the bath.

The influx of Na^+ and Cl^- is also dependent on the internal concentration of these ions. The influx for worms salt-depleted 3–4 days and placed in PW is elevated nine times for Na^+ and 2.6 times for Cl^-. This response is characteristic of most fresh-water animals. The mechanisms are not known but a neuroendocrine reflex may be involved (see below).

Active transport of Na^+ and Cl^- are largely independent processes. Sodium can be accumulated from Na_2SO_4 without the concomitant uptake of $SO_4^=$. This probably involves exchange with an endogenous cation such as NH_4^+ or H^+. Chloride can be accumulated from KCl probably in exchange for endogenous HCO_3^-. We have not analyzed these mechanisms but the rates of excretion of NH_4^+ and HCO_3^- are commensurate with the hypothesis.

When worms are transferred from soil to PW, ion and water balance are temporarily upset. Water is taken up and the animals gain weight for about two days but finally stabilize at about 115% of their weight in soil. The net uptake of H_2O initially is about 92 μl/10 g-hr which is a minimum estimate of the osmotic uptake of water under these conditions (Fig. 2).

Apparently the nephridia cannot immediately adjust to the increased uptake of water and the animals gain water. Inulin clearance in at least some (4 out of 10) soil-acclimated animals placed in PW was only 10 μl/10 g-hr or about eight times lower than PW adapted animals. Water balance is achieved when the osmotic force is balanced by hydrostatic force generated by the elasticity of the body wall plus the forces involved in eliminating water in urine and rectal fluid.

The net accumulation of water when transferred from soil to PW dilutes the solutes in the body fluids and activates the Na^+ and Cl^- pumps. In effect the animal behaves as if it is salt-depleted. This leads to a positive Na^+ and Cl^- balance and ultimately the concentrations of Na^+ and Cl^- in CF are restored to values almost identical with soil-animals. By varying the activity of the ion pumps rather than water content, these animals are able to adjust their ionic composition. This may reflect a direct effect of specific ions in the body fluids on exposed epithelia participating in transport or may involve a more elaborate neuroendocrine reflex mechanism analogous to what is found in vertebrates. Some evidence for the latter hypothesis has been provided by Kamemoto (1964) who reported a "brain factor" involved in water and salt balance in earthworms.

SUMMARY

1. Earthworms maintain a hyperionic steady state while living in a dilute solution containing Na^+, K^+, Ca^{+2}, Cl^- and HCO_3^- (PW).

2. The inside (coelomic fluid) of worms is electronegative by 12 to 30 mV relative to the bath (PW).

3. Sodium and chloride are transported across the skin against an electrochemical gradient. Each ion may be transported independently of the other presumably in exchange for an endogenous ion of like charge.

4. For animals in PW, Cl^- is exchanged at a rate of 0.9 μeq/10 g-hr and Na^+ at a rate of 0.3 μeq/10 g-hr. The influx of Na^+ is dependent on Na^+ concentration in the bath and displays saturation kinetics ($V_{max} = 1$ μeq/10 g-hr; $K_s = 1.3$ mMoles/l). Chloride kinetics were not analyzed.

5. Salt depletion increases the influx of Na^+ and Cl^- causing a net uptake of each ion.

6. Inulin and dextran are cleared from the coelomic fluid of PW-adapted worms at a rate of 75 μl/10 g-hr. Excretion of these compounds is probably through nephridia so that this is a maximum estimate of the rate of urine production. Water is also lost as a dilute rectal fluid which is formed at a rate of about 20 μl/10 g-hr.

7. Drinking of the bath occurs at a slow rate (4 μl/10 g-hr). The gut does not play a significant role in ion uptake from the bath but may be involved in the elimination of water. Possible mechanisms are discussed.

8. When worms are transferred from moist soil to PW they experience a net uptake and retention of water which dilutes their body fluids. Subsequently Na^+ and Cl^- influxes are elevated resulting in the net accumulation of Na^+ and Cl^- and, within seven days, the restoration of the original (soil) concentrations in the coelomic fluid (75 mM Na^+/l, 47 mM Cl^-/l).

9. Several lines of evidence indicate that hydromineral metabolism in worms is delicately controlled and involves the integrated activity of several organs. These control systems are discussed.

LITERATURE CITED

Adolph, E. F., 1927. The regulation of volume and concentration in the body fluids of earthworms. *J. Exp. Zool.*, **47**: 31–62.

Alvarado, R. H., and T. H. Dietz, 1970. Effect of salt depletion on hydromineral balance in larval *Ambystoma gracile*, II. Kinetics of ion exchange. *Comp. Biochem. Physiol.*, **33**: 93–110.

Bahl, K. N., 1945. Studies on the structure, development and physiology of the nephridia of oligochaeta. VI. The physiology of excretion and the significance of the enteronephric type of nephridial system in Indian earthworms. *Quart. J. Microscop. Sci.*, **85**: 343–389.

Boroffka, I., 1965. Elektrolyttransport im Nephridium von *Lumbricus terrestris*. *Z. Vergl. Physiol.*, **51**: 25–48.

Davson, H., 1964. *A Textbook of General Physiology.* Little, Brown, Boston, 1166 pp.

Dietz, T. H., L. B. Kirschner and D. Porter, 1967. The roles of sodium transport and anion permeability in generating transepithelial potential differences in larval salamanders. *J. Exp. Biol.*, **46**: 85–96.

Fretter, V., 1955. Uptake of radioactive sodium (^{24}Na) by *Nereis diversicolor* Mueller and *Perinereis cultrifera* (Grube). *J. Mar. Biol.*, **34**: 151–160.

Jørgensen, C. B., and R. P. Dales, 1957. The regulation of volume and osmotic regulation in some nereid polychaetes. *Physiol. Comp. Oecol.*, **4**: 357–374.

Jørgensen, C. B., H. Levi and H. H. Ussing, 1946. On the influence of the neurohypophyseal principles on the sodium metabolism in the axolotl (*Ambystoma mexicanum*). *Acta Physiol. Scand.* **12**: 350–371.

Kamemoto, F. I., 1964. The influence of the brain on osmotic and ionic regulation in earthworms. *Gen. Comp. Endocrinol.*, **4**: 420–426.

Kamemoto, F. I., A. E. Spalding and S. M. Keister, 1962. Ionic balance in blood and coelomic fluid of earthworms. *Biol. Bull.,* **122**: 228–231.

Krogh, A., 1939. *Osmotic Regulation in Aquatic Animals.* Cambridge University, London, 242 pp.

Laverack, M. S., 1963. *The Physiology of Earthworms.* Pergamon, Oxford, 206 pp.

Maluf, N. S. R., 1939. The volume- and osmo-regulative functions of the alimentary tract of the earthworm (*Lumbricus terrestris*) and on the absorption of chloride from freshwater by this animal. *Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere,* **59**: 535–552.

Maluf, N. S. R., 1940. Osmo- and volume-regulation in the earthworm with special reference to the alimentary tract. *J. Cell. Comp. Physiol.,* **16**: 175–187.

Morris, R., and J. M. Bull, 1968. Studies on freshwater osmoregulation in the ammocoete larva of *Lampetra planeri* (Bloch). II. The effect of de-ionized water and temperature on sodium balance. *J. Exp. Biol.,* **48**: 597–609.

Potts, W. T. W., and G. Parry, 1964. *Osmotic and Ionic Regulation in Animals.* Pergamon, Oxford, 423 pp.

Ramsay, J. A., 1949a. The osmotic relations of the earthworm. *J. Exp. Biol.,* **26**: 46–56.

Ramsay, J. A., 1949b. The site of formation of hypotonic urine in the nephridium of *Lumbricus. J. Exp. Biol.,* **26**: 65–75.

Robertson, J. D., 1936. The function of the calciferous glands of earthworms. *J. Exp. Biol.,* **13**: 279–297.

Roots, B. I., 1956. The water relations of earthworms. II. Resistance to desiccation and immersion, and behavior when submerged and when allowed a choice of environment. *J. Exp. Biol.,* **33**: 29–44.

Semal, P. van Gansen, 1959. Structure des glandes calciques d'*Eisenia foetida* Sav. *Bull. Biol. France Belgique,* **93**: 38–63.

Shaw, J., 1963. Kinetic aspects of ion regulation in aquatic animals. *Viewpoints Biol.,* **2**: 163–201.

Tillinghast, E. K., 1967. Excretory pathways of ammonia and urea in the earthworm *Lumbricus terrestris* L. *J. Exp. Zool.,* **166**: 295–300.

Ussing, H. H., 1949. The distinction by means of tracers between active transport and diffusion. *Acta Physiol. Scand.,* **19**: 43–56.

van Brink, J. M., and J. Rietsema, 1949. Some experiments on the active uptake of chlorine ions by the earthworm (*Lumbricus terrestris* L.) *Physiol. Comp. Oecol.,* **1**: 348–351.

Wolf, A. V., 1940. Paths of water exchange in earthworms. *Physiol. Zool.,* **13**: 294–308.

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

Reference: *Biol. Bull.*, **138**: 262–271. (June, 1970)

MATURATION OF THE GONADS AND SEASONAL CYCLE OF THE PLANKTONIC LARVAE OF THE OPHIUROID *AMPHIURA CHIAJEI* FORBES[1]

LUCIENNE FENAUX

Chargée de recherche au C.N.R.S. Station Zoologique, Villefranche-sur-Mer (06)

Amphiura chiajei is a little Ophiuroid known in the Mediterranean Sea and in the Atlantic Ocean; it is found from the coasts of Norway to Angola, also along the British Isles and Faroe and the Azores. It produces a planktonic larva, the development of which I have studied (Fenaux, 1963). Although this species is abundant in the Mediterranean, there are no previous records of the presence of its larvae. Only Müller (1853) describes an ophiopluteus from the Adriatic Sea, which seems to be related to *A. chiajei.*

In order to analyze the modalities of laying for a given species, it is as much important to know the conditions in which it takes place normally (and namely to precisely determine the periods during which the larvae appear in the plankton) as to follow the changes taking place during their gonad maturation. For this reason, in the course of my research on the ecological aspects of the reproduction of mediterranean Echinoids and Ophiuroids (Fenaux, 1968 b), I have studied the seasonal variations of gonads and the periodic presence of planktonic larvae of *A. chiajei.* These results appear in the present paper.

MATERIALS AND METHODS

For the study of maturation of their gonads, ophiuroids were collected twice a month in samples of black mud from the inner basin of Villefranche Bay between April 1965 and March 1966. The seasonal cycle of their ophioplutei was observed in the plankton hauls made regularly over a period of three years (April 1961–April 1964).

The number of male and female ophiuroids whose gonads were examined between April 1965 and April 1966 in either histological sections or smears is indicated below.

	A^{65}	M	J	A	S	O	N	D	J^{66}	F	M
Males	12	34	28	17	27	26	15	18	29	28	9
Females	14	27	16	18	21	18	10	14	20	10	21
Indeterminable	3	3							1		

The average size of the ophiuroids was 7 mm (diameter of the disc, arms not included); they were all dioecious. Gonads were examined either as smears or in 7 microns histological sections after fixation in the fluids of Bouin, Carnoy or

---

[1] This study is derived from my state doctor's thesis on the ecological aspects of the reproduction of Echinoids and Ophiuroids of Villefranche-sur-Mer, submitted in June 1968.

Flemming. Slides were stained with Alcian blue, Groat's haematoxylin, Cleveland or Metanyl yellow, or treated with the trichrome stain of Gabe and Martoja-Pierson (1957). These techniques have allowed me to follow the development of the gonads, the stages of which have been differentiated according to the classification adopted by Fuji (1960) for the sea-urchins *Strongylocentrotus nudus* and *S. intermedius.*

I have distinguished six stages of development which are identified as follows:

Stage 0. Gonads are very small and sex cannot be determined even after microscopic examination. I found this stage in seven ophiuroids collected in April and May 1965 and January 1966. These were *Amphiura* whose diameter did not exceed 4 mm (indeterminable ophiuroids mentioned above).

Stage 1. This is the stage which follows the shedding of sexual products. The gonads have a brown-red color. In males, spermatogonia line the wall of the follicle (Fig. 1 A). Within the gonad, one observes non-ejected spermatozoa, left over from the preceding maturation. In females, at the beginning of this stage, the oogonia and small primary oocytes are united in a tissue which is well stained by Alcian blue; this is the nutritive phagocytotic tissue. In certain cases, few large oocytes, remaining from the preceding maturation, can be observed inside the gonad (Figs. 1 A and B). At the end of this stage, the oocytes have invaded the ovary; they are small, 15 to 30 microns in diameter.

Stage 2 (Growth stage). In males, the spermatid layer is thick (Fig. 1 B). In females, there exist two types of oocytes: the first type whose average size is 30 microns and the second type, more numerous, whose average size is 60 microns (Fig. 2 C). On sections treated with the trichrome stain, the larger oocytes are paler and have a dark edge. This differentiation is equally evident in the sections stained with Alcian blue and Groat's haematoxylin. Moreover, the cytoplasm of the large oocytes shows the mesh-like appearance that Delavault (1960) described in the large oocytes of the asteroid *Echinaster sepositus.*

Stage 3 (Pre-maturation stage). In males, spermatogenesis is in progress. As has been observed in *E. sepositus* (Delavault, 1960), the growth of germinal tissue towards the center of the gonad appears as small columns (Fig. 1 C). In females, this stage is characterized by a large increase in the size of the oocytes (average diameter 130 microns). The secondary oocytes cannot be seen in the lumen of the acini (Fig. 2 D).

Stage 4 (Maturation stage). In males, the gonads are filled with spermatozoa (Fig. 1 D). In females, I never observed secondary oocytes on the numerous slides or smears examined. The problem of maturation in the females will be dealt with below.

Stage 5 (Shedding stage). In males, the non-ejected spermatozoa form a mass isolated from the wall of the gonad (Fig. 1 E). In females, I have considered as being in stage 5 those gonads which, although they do not show secondary oocytes, display an evolution in comparison with the pre-maturation stage. In fact, I observe in them a growth of primary oocytes; and in the spaces formed, most likely by the expulsion of a certain number of eggs, appears a vesicular tissue. In addition, certain large oocytes have a degenerating cytoplasm (Fig. 2 E).

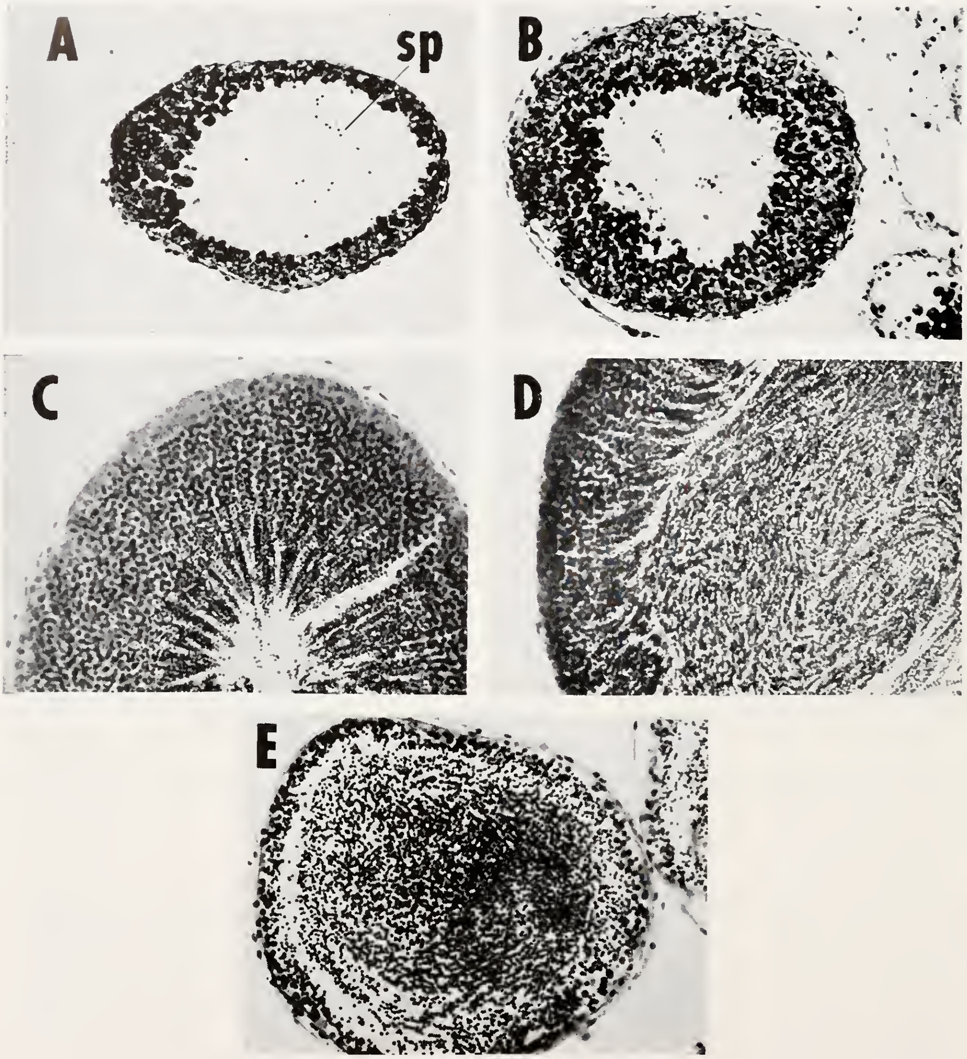


Figure 1. Histological sections of gonads of *Amphiura chiajei;* A. Stage 1 testis, in the center of the gonad, few spermatozoa not expelled (sp.), specimen collected in October 1965, × 300; B. Stage 2 testis, February 1966, × 300; C. Stage 3 testis, May 1965, × 120; D. Stage 4 testis, August 1965, × 120; E. Stage 5 testis, October 1965, × 120.

Seasonal Evolution of the Gonads

A. In males

Spring 1965. In April, of the 12 ophiuroids examined, 1 is at stage 1, 5 have gonads at stage 2 and in the remaining, spermatogenesis is in progress (Fig. 3 A). In May, nearly all the ophiuroids have arrived at the stage of maturity. As early as the month of June the stage 5 appears: it is not yet numerous.

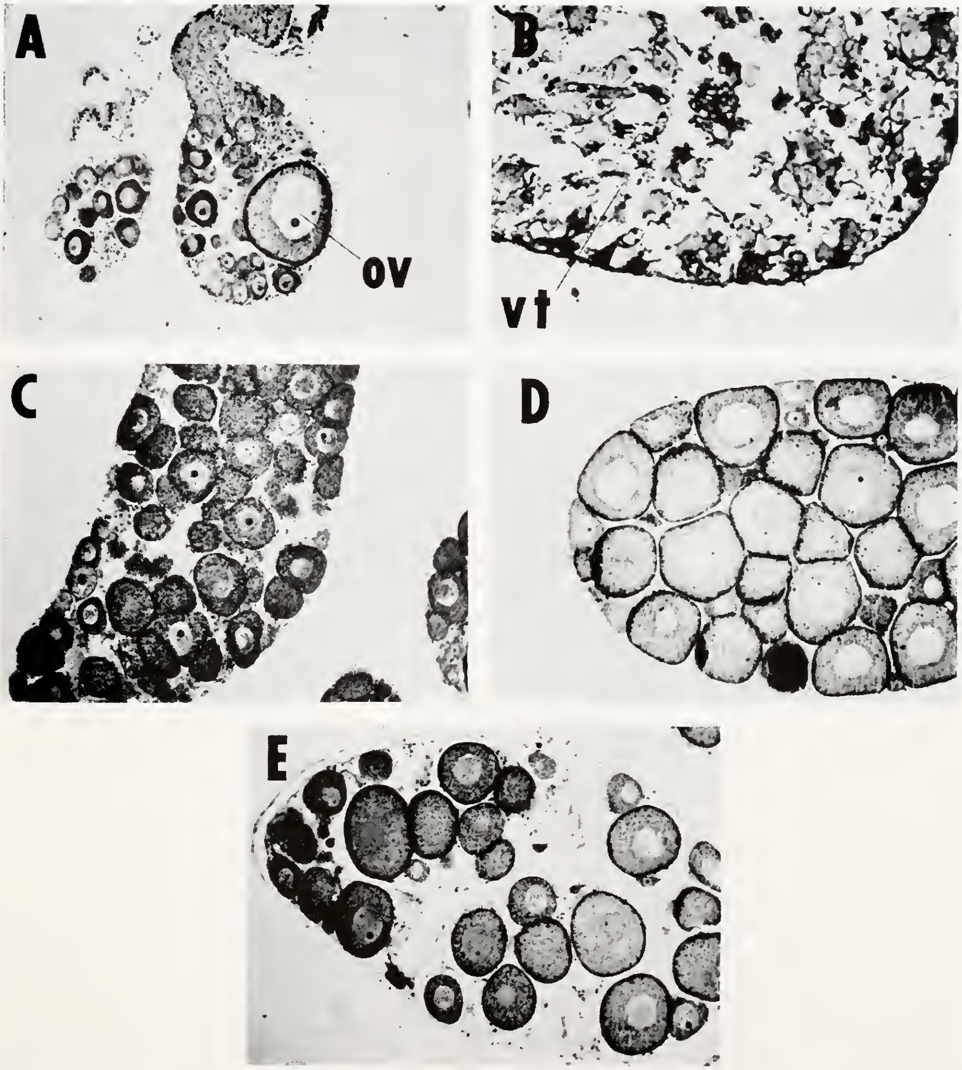


FIGURE 2. Histological sections of gonads of *Amphiura chiajei;* A. Stage 1 ovary, specimen collected in December 1965, ov = large oocyte remaining from the preceding maturation, × 300; B. Stage 1 ovary, October 1965, vt = vesicular tissue, × 300; C. Stage 2 ovary March 1966, × 120; D. Stage 3 ovary, June 1965, × 120; E. Stage 5 ovary, August 1965, × 120.

Summer 1965. There was no collection in the month of July. In August there are no more ophiuroids at stage 3, stages 4 and 5 are the only ones represented. Stage 5 is slightly more abundant than in June. In September, the majority of the ophiuroids are at the stage 4, some are at stage 1 and stage 5 is better represented than in August.

Autumn 1965. In October, stage 5 is the most abundant, stage 4 is still well represented and a few ophiuroids are at stage 1. In November and December the ophiuroids have gonads at stage 5, 1 and 2. Stage 5 which reaches a maximum in November, begins to regress in December and disappears completely by the end of February. Stages 1 and 2 become more abundant during autumn.

Winter 1966. The evolution observed since November is increased. Stage 1 reaches a maximum in February, then regresses in March. From February onwards, stage 3 is represented. It is predominant in March during which month some ophiuroids already reach maturity.

B. In females

Spring 1965. In April and May, stages 1, 2 and 3 are represented, but from May onwards a regression of stages 1 and 2 can be observed while the percentage of ophiuroids at stage 3 increases and reaches a maximum in June. As in males, some ophiuroids are at stage 5 at the end of spring (Fig. 3 B).

Summer 1965. During this season, stage 5 becomes most important. Some ophiuroids with gonads at stage 1 have been collected in August.

Autumn 1965. In October stage 3 is still well represented but it disappears in November. Stage 5, less abundant than in September, is still present until November. In December only stages 1 and 2 were observed.

Winter 1966. In January and February stage 1 is more abundant than stage 2. In March the two stages are in equal proportions.

The increase in size of the oocytes was followed by measuring in sections, the diameters of 50 oocytes according to the technique of Pearse (1965). The oocytes were classified in groups of ten microns (Fig. 4).

From April until June 1965, the average size of the oocytes increases progressively. In August it is slightly inferior to that of the month of June. This decrease in size is due to the fact that 35% of the ophiuroids are at stage 5 which is characterized by a loss of large oocytes and by a growth of young oocytes. In September, two modes are clearly distinguishable. The first mode is due mainly to females at stage 3, the second mode to females at stage 5, the stage during which there is growth of young oocytes. This situation is even more evident in October. Finally, in November, there is essentially only one mode in the frequency polygon: 80% of the females are at stage 1. The new generation of oocytes begins a period of growth which is almost linear until the month of March (Fig. 4).

In the males the appearance of stage 4 in the month of March indicates the beginning of the period of maturity and is followed shortly afterwards by the shedding period which begins in June, reaches a maximum in November and comes to an end at the beginning of winter. In the females the period of maturity probably begins in spring. As for males, stage 5 is present as early as June. As I already said, even if, in the gonads at stage 5 secondary oocytes are not seen, there is an evolution in comparison with the pre-maturation stage. This evolution is indicated by the appearance of vesicular phagocytotic tissue, the growth of a new generation of primary oocytes and the presence of large oocytes which have a degenerating cytoplasm. These characteristics suggest that the oocytes are expelled in a state of maturation. I shall discuss this possibility later. On the other hand, at the end of May and the beginning of June 1963, I observed egg-shedding in the laboratory. The embryos developed normally. It seems that the egg-shedding period begins in spring and finishes in November and thus, it is less extended than the period of sperm shedding.

SEASONAL CYCLE OF PLANKTONIC LARVAE

Few planktonic larvae of *A. chiajei* were collected: 116 in 710 hauls made at a depth of 5 meters at two stations of Villefranche Bay. The first station was at the entrance of the bay over rocky beds more than 50 meters deep and the second in the interior of the bay above a bed of a marine Phanerogam, *Posidonia oceanica,*

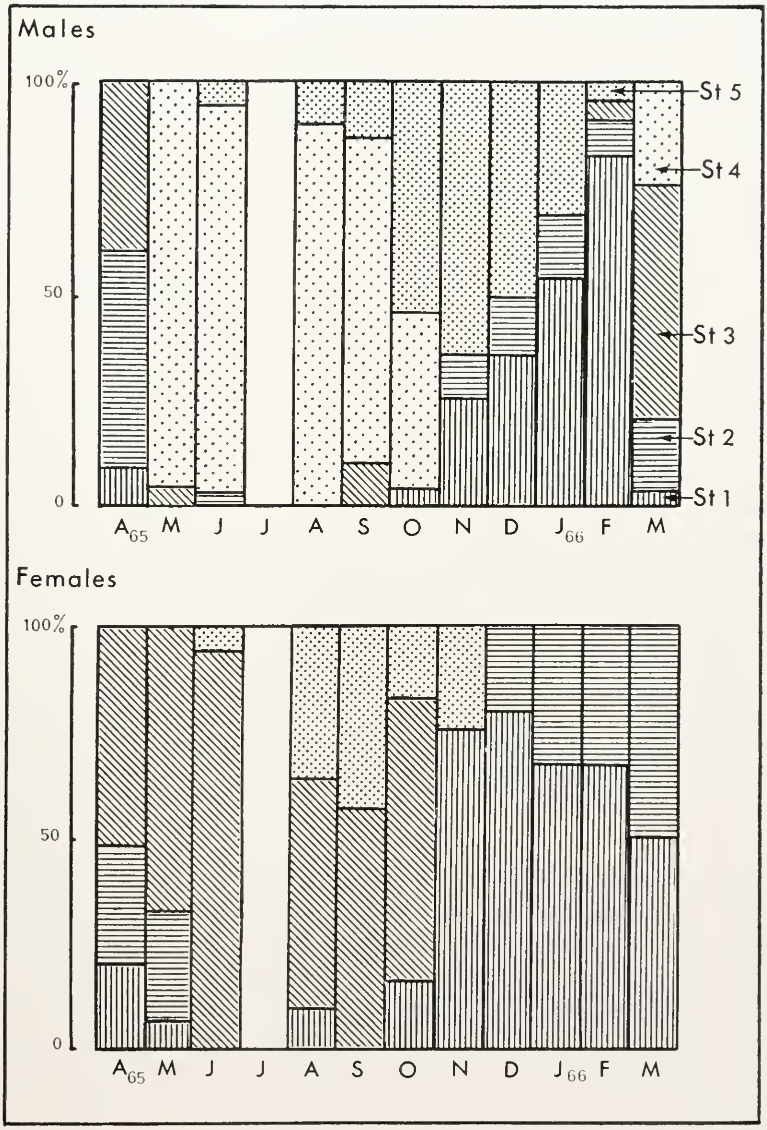


FIGURE 3. Relative proportion of animals in each gonad stage of *Amphiura chiajei.*

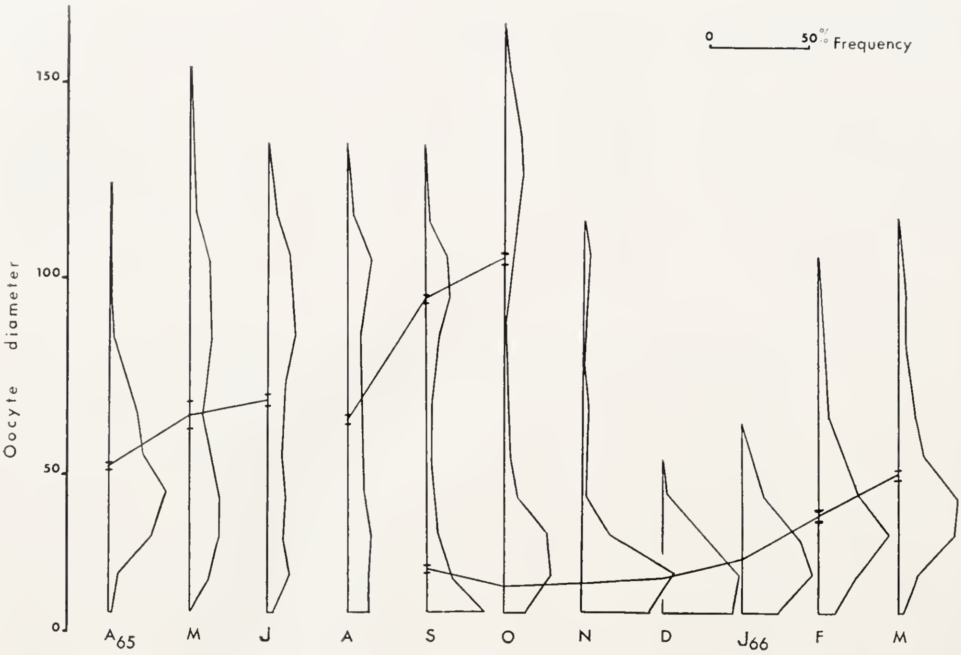


FIGURE 4. Evolution of the average size of oocytes (April 1965–March 1966).

25–30 meters deep (Fenaux, 1968 a). The annual temperature variation at this depth during the period studied was from 12° C to 16°C. Figure 5 represents the monthly averages for these four years. Autumn is the main period of the appearance of ophioplutei (Fig. 5). At station 1, one to two ophioplutei are found for ten planktonic hauls. At station 2, the number of ophioplutei harvested is greater because of the transport of larvae by currents from the abundant population of *Amphiura* in the interior basin of the bay. In August and especially in October and November quite large numbers of larvae are occasionally observed, more than ten per hauls, whereas the average catch is, as for station 1, one to three larvae for ten hauls. Several ophioplutei have been collected in winter and spring.

Nearly all the larvae collected were in the process of metamorphosis.

DISCUSSION

A histological examination of the gonads of *A. chiajei* has permitted me to state precisely the succession of the various stages of gametogenesis. After a period of sexual rest, the sex cells begin their development in winter (differentiation of the spermatids, growth of the primary oocytes) and come to maturity in spring. Reproduction takes place principally at the end of summer.

Concerning spermatogenesis, there is a relatively important phase of accumulation of spermatozoa. If the differentiation of spermatogonia into spermatids takes place during an extended period, such activity is only observed in a small percentage of the population.

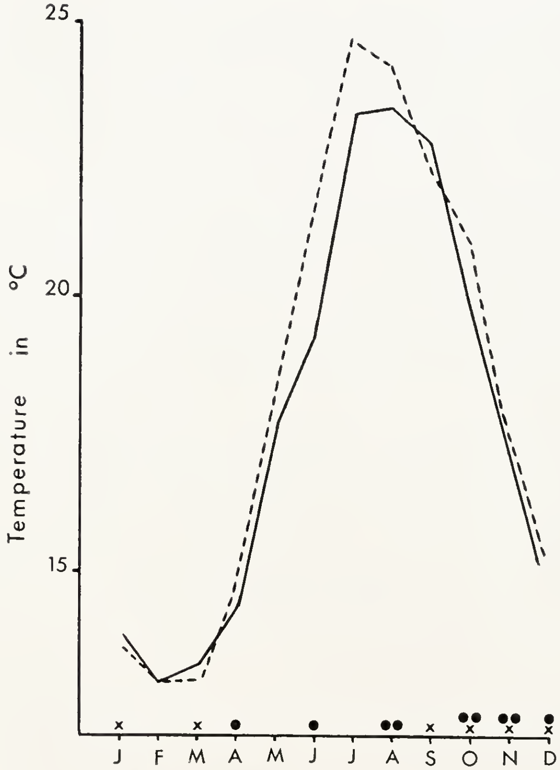


FIGURE 5. Presence of the *Amphiura chiajei* larvae in plankton hauls made at Station 1 (X) and Station 2 (●); X or ● = 1 to 2 ophioplutei for 10 planktonic hauls; ●● = 1 to 3 ophioplutei for 10 planktonic hauls; Continuous line curve: average temperature at 5 meters depth at Station 1 (January 1961–April 1964); Dotted line curve: average temperature at 5 meters depth at Station 2 (April 1961–April 1964).

As I pointed out above, although I have been able to observe the different stages of spermatogenesis, this is not true for oogenesis. In fact, no stage of maturation was disclosed in the numerous histological sections or smears which were examined. The shedding stages begin suddenly after a long period of primary oocytes growth. Moreover, the attempts made for artificial fertilization in June and in September, were unsuccessful. There was no expulsion of polar globules after the addition of spermatozoa, as was observed in the asteroid *Leptasterias hexactis* (Chia, 1968) or when the oocytes were liberated in water, as in the case of *Marthasterias glacialis.* It is probable that maturation is accomplished very rapidly within the gonad, and is completed only a short time before the ejection of the eggs. This would explain why the secondary oocytes are absent in the histological sections which were examined. Therefore, there is not, as in the males, a more or less protracted period of sexual maturation. This characteristic seems to be frequent in Ophiuroides; it was observed also, after a histological examination, in a Euryalae *Gorgonocephalus caryi* (Patent, 1969). Those in which artificial fertilization has been successful and which store their eggs for some time, are rare. Such rarity of secondary oocytes has been observed in other Echinoderms: *Odontaster validus* (Pearse, 1965), *Stylocidaris affinis* (Holland, 1967). According to Holland (1967), in Euechinoids and the Echinoderms apart from the Echinoids, after a very lengthy growth period of the primary oocytes, a rapid maturation occurs when the maximum size is reached. My observations on *A. chiajei* confirm this point of view.

Further information regarding the egg-shedding period is provided by the examination of plankton hauls. This period extends mainly from the end of summer until autumn; however, a few ophioplutei have been caught at the end of winter and in spring. Few planktonic larvae were collected. In the laboratory, I obtained a complete larval development, and the formation of a young ophiuroid whose larval appendages were completely reduced (Fenaux, 1963) within eight days at a temperature of 18°–20° C. Such brevity of larval life is probably the main cause of the poverty of ophioplutei in the plankton hauls. For the eggs laid at the end of summer and at the beginning of autumn in water in which the temperature is superior to 20° C, the pelagic life is probably even shorter.

I have described the larvae which develop from fertilized eggs obtained from a natural egg-shedding (Fenaux, 1963). They are identical to planktonic larvae: the differences in size between the planktonic ophioplutei and those reared in the laboratory are minimal. Only the post-lateral appendages are longer in planktonic larvae.

	Planktonic larvae	Larvae reared in lab.
Average length of body	260 microns	276 microns
Average length of post-lat. rods	472 microns	393 microns
Average length of somatic rods	88 microns	87 microns

It is possible to relate *A. chiajei* to a certain number of larvae previously described; for example the Bohuslän planktonic larva described by Mortensen (1920), who related it to *Ophiura affinis*. According to this author, color is the only notable difference between the ophiopluteus found at Bohuslän and those of the Adriatic described by Müller in 1853. Müller connected this larva with *A. chiajei* (which he called *Ophioplepis sundevalli*). I have compared Müller's drawings and the *A. chiajei* larva that I have reared, and, most probably, it is the same ophiopluteus. Chadwick (1914, page 184, Figures 46 and 46A of the Pl. III) reported larvae found in the plankton hauls carried out at Port Erin in the month of December. All the samples of this larva were in the process of metamorphosis. It seems that this larva also should be connected with *A. chiajei*. It shows numerous common characteristics with the ophiopluteus of this species, especially in junction of the somatic and the postero-lateral rods.

Taking these findings into account we can state precisely the periods of reproduction of *A. chiajei* in different regions. In Sweden, the egg-laying is in the middle of September (Mortensen, 1920) but it can be earlier, the larva described by Mortensen having been caught in August. In Port Erin, egg-laying probably takes place at the end of autumn. In the Mediterranean, at Naples, the period of maturity extends from May until September (Lo Bianco, 1909), at Villefranche in spring and in summer, with egg-shedding beginning at the end of summer.

Finally, in the Adriatic, the reproduction takes place in October (Vatova, 1950). This appearance of larvae at the end of summer in the Mediterranean Sea and in the Boreal areas shows that a determined temperature has not induced the maturation of eggs.

SUMMARY

1. The seasonal evolution of the gonads of *A. chiajei* during the period from April 1965 until March 1966 has been described. The reproductive cycle allows a period of sexual rest at the end of autumn, a period of growth in winter, a period of maturity in spring and in summer, and an egg-shedding period from the end of summer until the middle of autumn.

2. The seasonal cycle of planktonic larva was studied during a period of three years. The larvae appear in autumn; however, a few of them were caught in spring and in summer.

3. The problem of maturation of the primary oocytes is discussed. The larvae described by Müller (1853), Mortensen (1920) and Chadwick (1914) are related to *A. chiajei*. The periods of reproduction of this species in various Northern and Mediterranean regions are specified.

LITERATURE CITED

CHADWICK, H. C., 1914. Echinoderm larvae of Port Erin. *Proc. Trans. Liverpool, Biol. Soc.*, **22**: 467–498.

CHIA, F. S., 1968. The embryology of a brooding starfish Leptasterias hexactis. *Acta Zoologica*, **XLIX**: 321–364.

DELAVAULT, R., 1960. La sexualité chez Echinaster sepositus Gray du Golfe de Naples. *Pubbl. Sta. Zool. Napoli*, **32**: 41–45.

FENAUX, L., 1963. Note préliminaire sur le développement larvaire de Amphiura chiajei Forbes. *Vie Milieu*, **XIV** (1): 91–96.

FENAUX, L., 1968 a. Maturation des gonades et cycles saisonniers des larves chez Arbacia lixula, Paracentrotus lividus et Psammechinus microtuberculatus (Echinides). *Vie Milieu*, **XIX** (1 A): 1–52.

FENAUX, L., 19628 b. Aspects écologiques de la reproduction des Echinides et Ophiurides de Villefranche-sur-Mer. Thèse d'état.

FUJI, A., 1960. Studies on the biology of the sea urchin. I. Superficial and histological changes in gametogenic process of two sea urchins, Strongylocentrotus nudus and S. intermedius. *Bull. Fac. Fish. Hokkaido Univ.*, **11**, (1): 1–14.

GABE, M., AND MARTOJA-PIERSON, M., 1957. Sur une coloration trichrome en un temps sans différenciation. *Bull. Microsc. Appl.* **7**: 60–63.

HOLLAND, N. D., 1967. Gametogenesis during the annual reproductive cycle in a cidaroid sea urchin (Stylocidaris affinis). *Biol. Bull.*, **133** (3): 578–590.

LO BIANCO, S., 1909. Notizie biologiche riguardante specialmente il periodo di maturità sessuale degli animali del Golfo di Napoli. *Mitt. Zool. Sta. Neapel*, **19** (4): 556–566.

MORTENSEN, TH., 1920. Notes on the development and larval forms of some Scandinavian Echinoderms. Vidensk. Medd. Dansk. *Naturhist. Foren. Kjobenhaun*, **71**: 133–160.

MÜLLER, J., 1852 (1853). Über den allgemeinen Plan in der Entwickelung der Echinodermen. *Abh. Deut. Akad. Wiss. Berlin*, **1853**: 123–219.

PATENT, D. H., 1969. The reproductive cycle of Gorgonocephalus caryi (Echinodermata; Ophiuroidea). *Biol. Bull.*, **136**: 241–252.

PEARSE, J. S., 1965. Reproductive periodicities in several contrasting populations of Odontaster validus Koehler, a common antarctic asteroid. Biology of the Antarctic Seas. II. *Antarctic Res. Series* **5**: 39–85.

VATOVA, A., 1950. Gli Echinodermi delli Laguna Venta. *Nova Thalassia*, **I** (7): 3–13.

Reference: *Biol. Bull.*, **138**: 272–285. (June, 1970).

BODY TEMPERATURE AND SINGING IN THE KATYDID, *NEOCONOCEPHALUS ROBUSTUS* (ORTHOPTERA, TETTIGONIIDAE)

JAMES EDWARD HEATH AND ROBERT K. JOSEPHSON

Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801,
Department of Biology, Case-Western Reserve University, Cleveland, Ohio 44106
and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Insects in the family Tettigoniidae produce sound by rubbing a sclerified ridge (plectrum) on the medial edge of the right forewing across a set of teeth (pars stridens) on the underside of the left forewing (Dumortier, 1964). The songs produced are composed of chirps, each chirp produced by a movement of the wings by one another. In some tettigoniids the chirp frequency is high enough that the sound produced appears continuous to a human listener (Pierce, 1948; Alexander, 1956). The frequency of wing movements and of chirps during singing in the katydid *Neoconocephalus robustus* is 150–200 per second (Josephson and Elder, 1968). One expects from this extraordinarily high frequency that the muscles moving the wings must have very high metabolic rates during singing, and that the thoracic temperature may rise well above the ambient temperature. The expectation of a high metabolic rate is in part confirmed by the structure of the wing muscles; the mitochondria in these muscles are large and numerous and comprise about 44% of the muscle fiber volume (Josephson and Elder, 1968). In this paper we will show that the thoracic temperature during singing is indeed considerably greater than the environmental temperature and that the katydid uses physiological and behavioral means to achieve elevated temperatures. Measurements of the thoracic temperature allow estimates of the metabolic cost of singing.

Heat production resulting from muscular activity and temperature regulation have been studied in a number of insects, usually in connection with flight and pre-flight warmup (Heath and Adams, 1967). *N. robustus* offers an unusually favorable preparation for examining temperature relations during intense muscular activity in an intact insect. The animals will sing spontaneously even when enclosed in a small container. Electrical measurements and temperature recordings can be made from an animal without resorting to either elaborate tethers or wind tunnels generally necessary to adequately study flying insects. Further the onset and cessation of singing are under the control of the animal rather than the experimenter as is often the case in flight experiments. The regulation of temperature by the regulation of activity is therefore a mechanism available to the animal.

MATERIALS AND METHODS

Adult specimens of *N. robustus* were collected from salt marshes in the vicinity of Woods Hole, Massachusetts. Only males sing and only males were used in these studies. The animals were kept in the laboratory under approximately normal diurnal fluctuation in light intensity and were fed lettuce. Most animals sang spontaneously at or shortly after sundown each night. Males weigh 0.4–0.5 grams.

Muscle action potentials were recorded with 50 μ silver wires, insulated except at the tip. The electrodes were inserted into the desired muscles through small holes in the exoskeleton and were sealed in place with dental wax. The indifferent electrode was a bare silver wire in the abdomen. The electrode positions were verified by post-mortem dissection. Capacitor-coupled amplifiers were used to amplify the potentials which were then stored on magnetic tape for later photography and analysis. Both 40 gauge (0.2 mm) copper-constantan thermocouples and ultra-small thermistors (0.25 mm) were implanted in the thorax. Thermocouples were introduced through a hole punched in the pronotum and angled ventrally and posteriorly to lie within the mesothorax about 3 mm from the dorsal surface.

The animals were anesthetized with CO_2 while mounting recording electrodes and temperature transducers. Most animals did not sing in the evening following electrode and transducer implantation but they generally did on subsequent nights.

The thoracic temperatures were obtained from animals in the field and during behavioral studies by a specially constructed thermistor probe described by Heath (1967) and Heath and Adams (1969). Insects were captured in a net or grasped between the fingers by the posterior end of the elytra. The thermistor probe was punched through the dorsal surface to a depth of 4 mm. This probe has a thermal time constant of 0.2 sec. If the time between capture and measurement exceeded 5 seconds, the measurement was discarded because of the rapid heat loss from the insect.

RESULTS

Muscle activity during warm-up and song

Singing does not begin abruptly but is preceded by a period of muscle activity appropriately called warm-up, since it coincides with a noticeable rise in thoracic temperature. During warm-up the wings lie against the body and are not held in the slightly raised position taken during singing. There is little obvious movement of the thorax or wings during warm-up but the muscle activity is clear from electrical recordings of muscle action potentials. Initially the warm-up activity is discontinuous, occurring in bursts each several seconds long. These bursts become longer and more frequent and during late warm-up the activity is nearly continuous. There is usually a brief pause in muscle activity at the transition between warm-up and song.

Mortality was rather high in those animals implanted with both recording electrodes and a temperature transducer. Satisfactory records were obtained from only one animal. The results from this animal will be considered in some detail, however, since both the muscle activity and the thoracic temperature records are characteristic of all animals in which these parameters were measured independently. The action potential frequency from one of the forewing muscles of this animal during warm-up and singing is shown in Figure 1. During both late warm-up and singing the frequency of muscle action potentials is nearly constant

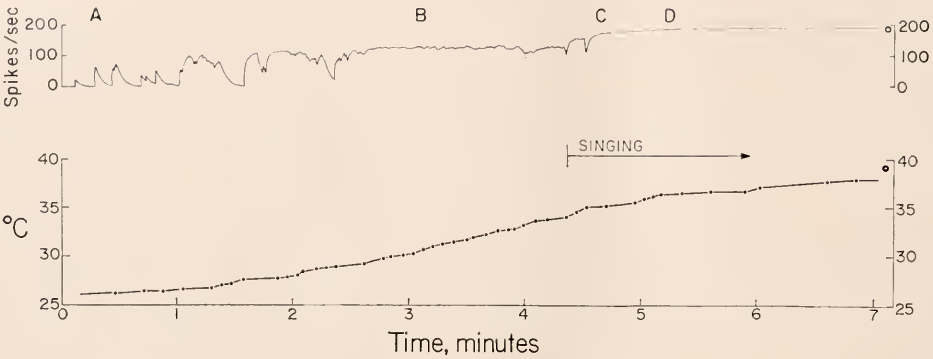


FIGURE 1. Muscle action potential frequency (right tergosternal muscle of the mesothorax) and thoracic temperature during warm-up and song. The recorded action potentials triggered a pulse generator whose output was fed into a leaky integrating circuit made with an operational amplifier. The triggering level was adjusted so that all large potentials, including the individual pulses in bursts triggered the pulse generator. The upper curve is the output of the integrator. Following a sudden change in pulse frequency the integrator output exponentially approaches an asymptote. The asymptotic value is a linear function of pulse frequency and the time constant of the approach is approximately 2 secs. The open circles at the right indicate spike frequency and thoracic temperature at the end of this singing period, approximately 4 minutes after the end of the records shown. The letters above the upper curve indicate the portions of the sequence from which the records of Figure 2 were taken.

even though the thoracic temperature steadily increases. This suggests that the pacemakers which generate the action potential patterns may be located in some part of the animal which does not undergo the large temperature changes seen among the musculature of the mesothorax.

Some details of the muscle electrical activity are seen in Figure 2. During singing the action potentials in the two recording channels are out of phase (Fig. 2D) indicating that the muscles, the right subalar and tergosternal, are antagonists during singing. At the onset of singing the tergosternal (lower channel) sporadically failed to fire for one or sometimes several consecutive cycles (Fig. 2C). This skipping of occasional cycles gives an opportunity to examine electrical interactions between the recording channels. Because of the small size of the thorax and the relatively large volume of the active muscles, electrical isolation between recording channels is necessarily incomplete. Some part of the signal recorded with extra-cellular electrodes in one muscle is inescapably due to activity in surrounding muscles. During missed cycles the record from the tergosternal muscle shows a small deflection coincident with the action potential from the subalar (Fig. 2E). This represents pickup from the subalar and its synergists. In *N. robustus* synergistic muscles fire essentially synchronously during singing. This could hardly be otherwise, given that the whole wing movement cycle is completed within 5–6 msec. In skipped cycles there is no deflection in either the tergosternal or the subalar records at the expected time of tergosternal firing. This suggests that not only the right tergosternal muscle but also all or most of its synergists have not fired; otherwise, one would expect to see electrical events generated by adjacent muscles in the records at this time. There is some

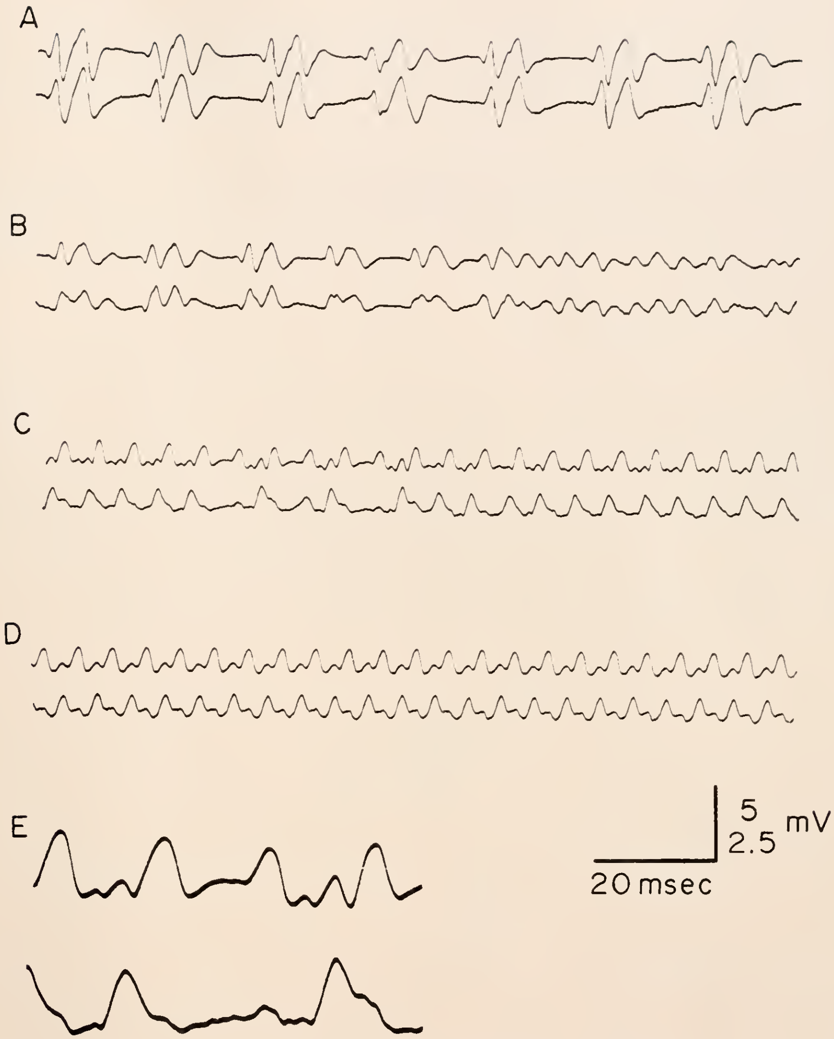


FIGURE 2. Action potentials from the right subalar (upper channel) and the right tergosternal (lower channel) muscles of the mesothorax. A portion of C, including a cycle in which the tergosternal failed to fire, is enlarged 3 times in E. The calibration applies to A-D.

pickup from antagonistic muscles in each channel, but the major deflections are clearly produced by the muscle from which the recording is made with possible contributions from its synergists.

During early warm-up, potentials in each channel are essentially synchronous. Since electrical coupling between recordings from antagonistic muscles has been shown to be small, this indicates that muscles which are antagonists during singing contract simultaneously during warm-up. Similar multichannel recordings from other animals indicate that muscles synergistic during singing maintain their synchrony during warm-up as well. Thus, during warm-up there is synchronous activity among a number of the forewing muscles. Since the active muscles include a number of antagonistic elements the resultant is heat production with little overt movement.

During early warm-up the muscle action potentials usually occur as pairs or short bursts of pulses. The interval between adjacent pulses can be quite short. The average interval between the peaks of the pulse pairs of Figure 2A is only 4.3 msec. During later warm-up the muscle activity becomes faster but less regular (Fig. 2B).

Body temperature during activity

Eight males were implanted with thermocouples and their thoracic temperature monitored for 3–5 successive nights. Figure 3 shows a sequence of warm-up and singing for a single male.

Activity begins with a period of warm-up. The increase in temperature during warm-up is approximately linear. Toward the end of the warm-up period the rate of warming may decline. When singing begins, the rate of warming is further decreased. During the singing period the body temperature may rise an additional increment. When singing stops, the body temperature falls rapidly along a Newtonian course back to ambient temperature.

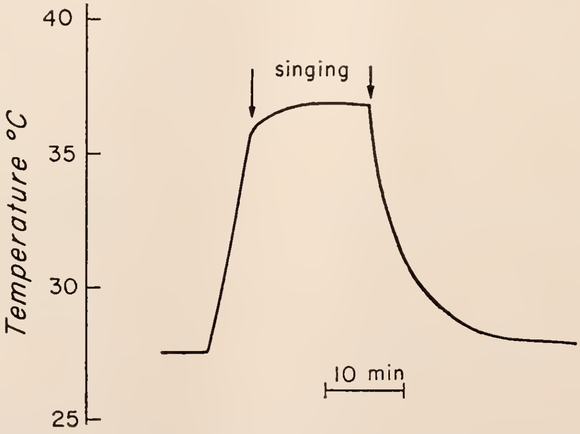


FIGURE 3. Thoracic temperature during a singing period of *N. robustus*. Air temperature was 27.5° C.

Warm-up. The rate of rise of thoracic temperature is nearly constant for a given warm-up period. It varies from 0.94° C/min to 2.20° C/min. Thirty warm-ups from eight animals averaged 1.54° C/min (± S.D. 0.33). These periods occurred at ambient temperatures from 22.5° C to 29° C.

Singing. Animals in the laboratory began singing at an average thoracic temperature of 33.5° C (S.D. 1.48; N = 23). The thoracic temperature at the onset of singing increased with air temperature 0.65° C/°C over the range 23.5°–

29° C. The lowest thoracic temperature at which singing began was 30.5° C, which occurred at ambient temperatures of 27.6° C and 28.5° C.

During singing thoracic temperature rises by about 1° C, reaching an average temperature of 34.4° C (S.D. 2.25; N = 30). The maximum thoracic temperature during singing increases with increasing ambient temperature. A linear regression fitted to data collected in the laboratory had a slope (thoracic temperature/ambient temperature) of 0.60° C/°C over the temperature range 22.5–29.5° C (Fig. 4). Katydids can sing at lower air temperatures in the field. Temperatures of katydids singing at air temperature of 16.8° C–22° C in the field are also shown in Figure 4. The linear regression fitted to the laboratory data seems to fit the field measurements. This katydid may be unable to sing at thoracic temperatures below about 28° C.

Singing period. Neoconocephalus robustus sings readily in the laboratory for a period of up to an hour even when implanted. In the field this katydid sings continuously for extended periods. Table I shows the characteristics of the song recorded during several evenings near Falmouth, Massachusetts in August, 1968. On subsequent evenings we found *N. robustus* singing at air temperatures as low as 16.8° C and also as late as 10:00 PM (EST).

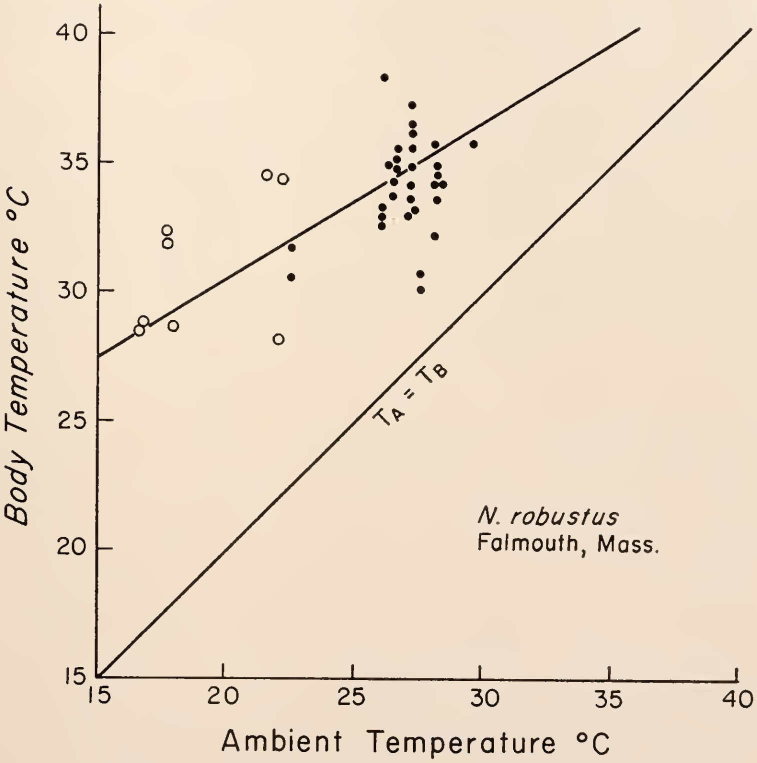


FIGURE 4. Thoracic temperature of singing katydids plotted against air temperature at the time of singing. A linear regression fitted to points from animals measured in the laboratory, fits field measurements, ○, equally well.

TABLE I

Duration and conditions during singing activity by N. robustus

Animal	Range of call duration	Sum of call periods	(N) calls	Time EST	Date	Air temp °C
A	37″– 9′27″	38′26″	7	7:06–10:05	8/13	—
B	5′00″–51′42″	70′09″	5	7:13– 8:23	8/15	21°
C	9′59″–32′37″	53′45″	3	7:38– 8:39	8/15	21°
D	39′26″	39′26″	1	8:01– 8:40	8/23	23°
E	3′00″–68′19″	80′11″	4	7:20– 8:45	8/23	23°
$\bar{X}$	37″–68′19″	56′25″	20			

Cooling. At the end of an active period the thoracic temperature returns to ambient levels. The cooling rate is approximately linear with temperature gradient and is thus passive or Newtonian (Fig. 5). The cooling curve can be used to estimate heat loss in a given gradient or heat production if the body temperature is constant.

Behavioral studies

This phase of the study was intended to determine the thermal limits of activity and to locate reliable behavioral estimates of the temperature tolerance

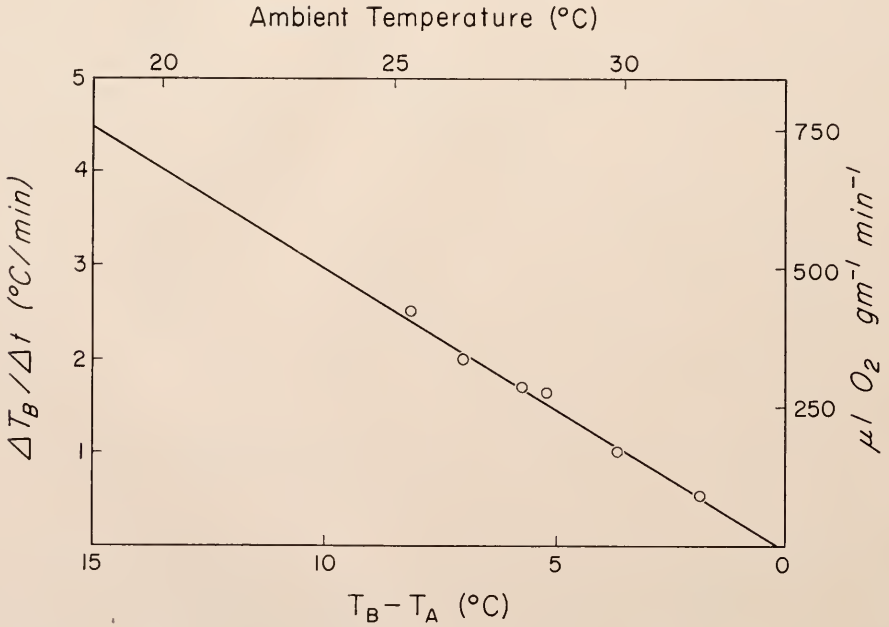


FIGURE 5. Cooling curve of the thorax plotted as a rate of change of temperature (left axis) against gradient (bottom axis). A line passing through zero is fitted by eye to the points. If the insect maintains a thoracic temperature of 33.5° during singing and temperature change is converted to heat production (right axis), then the line is an estimate of the expense of singing at decreasing ambient temperatures (upper axis). (See discussion for further explanation.)

of this animal. In each case, males captured in the field, in the evening, were brought into the laboratory and investigated the next day.

Response to cold. Individual males were cooled in beakers immersed in an ice bath until immobilized. They were then removed to room temperature (~25° C), and allowed to warm.

As the insects warmed, responsiveness returned. When the insect could right itself, its thoracic temperature was quickly recorded. This response appeared at an average body temperature of 21.8° C (Table II). This is an unexpectedly high value since individuals in nature sing at lower air temperatures. Some individuals retain awkward and limited motor activity during cooling to as low as 11° C, but once immobilized motor control does not reappear during warming until 21° C is reached.

Table II

Behavioral responses to temperature in Neoconocephalus robustus

Response	Mean (°C) (S. D.)	Range (°C)	N.
Righting	21.8 (1.24)	19.5–23.5	10
Voluntary flight	33.7 (2.30)	29.0–35.2	5
Shade-seeking	37.6 (1.60)	35.0–40.0	10
Loss of motor control	43.9 (0.64)	42.5–44.5	10

Response to heat. Individual males were heated in pasteboard containers held under a 250 watt infra-red heat lamp until they lost motor control. They were then quickly removed and the thoracic temperature recorded.

The loss of motor control occurs at an average temperature of 43.9° C (Table II). This temperature is an ecological lethal point because individuals in nature would be unable to avoid further increases in body temperature by seeking cooler locations once this level is passed. All individuals regained control upon cooling, but two of twelve individuals treated in this manner died within 24 hours.

Maximum voluntary tolerance

Individuals in the previous experiment became exceptionally active during the later stages of heating. This activity is equivalent to the behavioral response of heat avoidance or shade-seeking. Insects in the field periodically are exposed to the direct rays of the sun. Insects typically retreat from these positions either upon first contact with direct irradiation or at a closely regulated temperature level after some minutes of exposure (Heath, 1969a). *Neoconocephalus robustus* follows the latter course.

To determine the temperature level of this response, individuals were placed on a segment of pine branch 18 cm below 250 watt IR lamp. The animals warmed for several minutes then shifted to a shaded position beneath the branch. They were then quickly retrieved and thoracic temperatures measured. These movements occurred at average thoracic temperatures of 37.6° C (Table II).

During the warming *N. robustus* takes up basking positions maximizing the surface area presented to the radiant source. If the insect is replaced on the branch in a shaded position following measurement it returns shortly to a fully exposed position beneath the lamp. Ultimately it takes up a partially exposed position on the branch and remains indefinitely at or slightly below the temperature of shade-seeking. The persistance of these patterns is strong evidence that during the daylight hours, *N. robustus* regulates its body temperature behaviorally to the same range of temperature that males achieve in the evening while singing.

A few individuals flew rather than moved to shade. This response appeared at 33.7° C (Table II). Attempts to force flight by throwing individuals into the air at ambient temperatures of 20°–30° were unsuccessful.

Discussion

The katydid, *Neoconocephalus robustus* has a very distinctive and intensive song. The song is generated by wing movements driven by muscles at an exceptionally high frequency. During singing this insect not only generates enough heat to raise the thoracic temperature well above ambient but it may depend upon reaching an elevated temperature in order to achieve the wing frequency necessary for its song. It precedes singing with a period of warm-up during which it contracts the thoracic muscles without significant movement. This is accomplished by firing antagonist muscles synchronously. The only product of this activity is heat which causes the thoracic temperature to rise. The pattern of muscular activity changes abruptly when an elevated thoracic temperature is reached and singing begins.

Heat production due to muscular exertion is well known among insects (Sotovalta, 1954). The locust, *Schistocerca gregaria*, may warm 6° C during flight (Weis-Fogh, 1952). The cicada, *Magicicada septendecem*, may sustain a 2–6° increment above ambient temperature by intermittent flight (Heath, 1968b). In neither case do they seem to depend or capitalize on this heat production in normal activity. On the other hand, some insects, notably bees and moths use the heat generated by flight to maintain relatively constant thoracic temperatures during activity (Esch, 1968; Heath and Adams, 1967). In the case of moths a period of warm-up precedes flight. As in the katydid, antagonist muscles contract synchronously yielding little movement but high heat production (Kammer, 1968; McCrea, 1969). Both bees and moths are conspicuously insulated to retard heat loss (Church, 1960), and among moths the suite of behavioral, physiological and morphological adaptations permit these animals a degree of independence of environmental conditions of significance to a nocturnal animal.

The katydid, *N. robustus*, is intermediate between passive heat producers like the locust and active endothermic insects like the moth. The katydid lacks an insulative layer to retard heat loss. It also is more sensitive to ambient temperature during heat generating activity than moths. Over the range of ambient temperatures it is likely to encounter in nature (15°–30°), its thoracic temperature during singing shows a positive regression with ambient temperature of 0.6° C/°C. Moths over the same range are independent of air temperature (Heath & Adams, 1967). Apparently, katydids depend on heat production only for singing while many moths feed, seek mates, lay eggs only when the body temperature is regulated to a controlled range (Heath and Adams, 1967). The katydid does not depend upon internal heat production during feeding, mating and egg laying. On the other hand, *N. robustus* like many diurnal insects will bask readily when presented with a source of radiant energy. Its behavior predicts that during daylight hours it conducts its activities over a range of body temperatures similar to those achieved by singing. Figure 6 summarizes the thermal responses of *N. robustus*.

Both the rapidity of the muscle activity and the large gradient maintained between the thorax and surroundings predict that the male katydid must expend energy at an extraordinary rate during singing. During a singing period the body temperature remains relatively constant, which means that the heat produced just balances the heat lost. That portion of the muscular activity that produces heat at a given gradient to the environment can be estimated exactly as the rate the body cools through the same gradient.

If heat production during cooling is small compared to heat loss, if the convective flow about the singing insect is disregarded and if the singing process is inefficient (*i.e.*, most of the expended energy appears as heat rather than sound), then analysis of the cooling curve of this insect can be used to estimate the cost of singing.

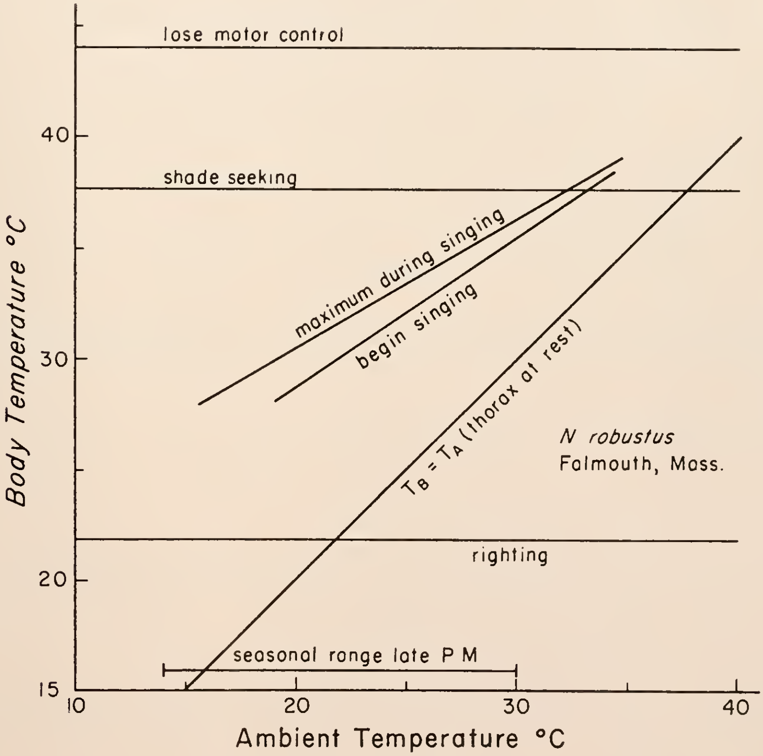


FIGURE 6. Summary of the temperature responses and states of *N. robustus*. The responses are explained in text. A bar representing the air temperatures ordinarily encountered during the singing season in the field is shown at the bottom of the graph.

It has been shown that the metabolic rate during flight of insects is often 100 times that of the resting rate (Prosser and Brown, 1961). Hence, heat production during cooling should be very small. The singing katydid does generate a flow of air about the thorax which is not present in the quiet insect. Hence, the cooling curve will underestimate heat production of singing. Similarly, the cooling curve will also underestimate the energy dissipated as sound and kinetic transfer during singing. It is unlikely that the katydid is more than 10–15% efficient. Heath and Adams (1967) showed that metabolic rate during activity in sphinx moths exceeded the rate calculated from the cooling curve by about 15%. Hence, the cooling curve can be used to make a conservative estimate of metabolic rate in singing.

The rate of temperature change with time for an object such as an insect's thorax which produces heat internally and loses it to the environment is given by the following:

$$\frac{dTB}{dt} = \frac{1}{WS}\left(\frac{dHp}{dt} - \frac{dH1}{dt}\right) \tag{1}$$

$\frac{dTB}{dt}$ = rate of change in body temperature (deg/sec)

W = mass of object (g)

S = specific heat of object (cal/g deg)

$\frac{dHp}{dt}$ = rate of heat production (cal/sec)

$\frac{dH1}{dt}$ = rate of heat loss (cal/sec)

If it is assumed that heat loss is due entirely to conduction from the object's surface to the environment, the rate of heat loss is given by Newton's law of cooling:

$$\frac{dH1}{dt} = AC(T_B - T_A) \tag{2}$$

A = surface area of object (cm^2)

C = thermal conductance of the surface (cal/deg sec cm^2)

T_B = body temperature (deg)

T_A = environmental temperature (deg)

Solving (1) for the rate of heat production and substituting (2) for the rate of heat loss gives:

$$\frac{dHp}{dt} = WS\frac{dTB}{dt} + AC(T_b - T_a) \tag{3}$$

At the cessation of singing heat production is presumably negligibly small. Under these conditions, when dHp/dt is essentially zero, equations (1) and (3) can be rewritten as:

$$\frac{dH1}{dt} = -WS\frac{dTB}{dt} \tag{4}$$

$$\frac{dTB}{dt} = \frac{AC}{-WS}(T_B - T_A) \tag{5}$$

From (5) the rate of cooling at the cessation of singing is a linear function of the temperature gradient between the animal and its environment. In Figure 5 this is plotted for an animal of known weight using the method of Heath and Adams (1969). A line intersecting zero gradient was fitted by eye to the points. Using thoracic weight and assuming a specific heat of 0.8 for insect tissue (Krogh and Zeuthen, 1941) the heat loss for each gradient is readily calculated from equation (4) (Table III). This value equals the heat production necessary to maintain the same gradient. These were converted to the more familiar measurement of weight-specific oxygen consumption by assuming a caloric equivalent of oxygen of 4.8 cal/ml O_2. The high metabolic rate predicted by these calculations is consistent with both the muscular activity and poor insulation. For an animal to maintain a temperature gradient of 15° C between its thorax and the environment it must produce approximately 0.5 cal/min which is equivalent to an oxygen consumption of 0.1 ml O_2/min or, for a 0.14 g thorax, 0.75 ml O_2/g thorax wt/min. This is similar to the O_2 consumption values reported for actively flying insects which range from 0.17 to 1.5 ml O_2/g body weight/min (Prosser, 1961).

Another means of dramatizing the turnover of energy in a singing katydid is to estimate the rate of disappearance of metabolites with activity. If we assume a 15° C gradient, a singing *N. robustus* would expend about 30.6 cal/hr. This would require 0.0032 g of fat at 9500 cal/g. At a total weight of 0.4 g, *N. robustus* metabolizes 0.80% of its body wet weight in one hour of singing (or, assuming a water content of 80%, 4% of its dry weight). These figures underestimate expended energy, because some energy appears as sound and movement of the wings.

During much of warm-up and during singing the body temperature increases approximately linearly with time. When rate of rise of body temperature and

TABLE III

Energy expenditure of N. robustus *during singing*

$T_B - T_A$	cal/min*	ccO_2/g/min*	Fat consumption		
			g/hr	% wet body wt**	% dry body wt**
5°	0.17	0.25	0.0011	0.27	1.4
10°	0.34	0.50	0.0021	0.53	2.9
15°	0.51	0.75	0.0032	0.80	4.0

* Heat production by thorax only (0.14 g).

** % Total body wt (0.40 g wet; 0.08 g dry).

the ambient temperature are constant, the rate of heat production is a linear function of the body temperature (equation 3). During late warm-up and early singing, when the body temperature is linearly rising and hence the rate of heat production increasing, the frequency of muscle contractions is nearly constant for long periods. Thus it appears that the amount of heat liberated by each contraction increases as the body temperature increases. The muscle action potentials give no indication of increasing recruitment of muscles or units within muscles with increasing thoracic temperature so the greater heat production probably reflects greater energy release by the same population of muscle fibers. The duration of the twitch initiated by each action potential presumably becomes shorter as the thoracic temperature increases (*cf.* Neville and Weis-Fogh, 1963). The rate at which energy is released during a twitch must therefore have a rather high temperature coefficient for the heat production per contraction to increase in the face of shortening twitch duration.

N. robustus by the mechanism cited generates a distinctive song at considerable expense. The cost of the song is met perhaps because of competitive interaction with another species of *Neoconocephalus* in nearly the same habitat. In the study area *N. robustus* overlaps broadly with *N. ensiger.* The two species are very similar in appearance, but *N. ensiger* powers its rather soft intermittant song by much slower muscles (10–15 cps). Among tettigoniids song distinctiveness is strongly selected. Some insight into this might be derived by careful comparative cost analysis of *N. ensiger* and *N. robustus* song.

This work was supported in part by NSF Grant GB-6303 to J. E. H., PHS Grant NB-06054 to R. K. J. and by Grant GB-3447 from the National Science Foundation to the Department of Invertebrate Zoology, Marine Biological Laboratory. We give special thanks to Maxine Heath, R. Halverson, and N. Lazarow for their assistance.

SUMMARY

1. The katydid, *Neoconocephalus robustus*, produces a continuous song by rubbing its forewings together at frequencies of 150–200 per second. During singing the thoracic temperature is 5–15° C higher that that of the environment, the temperature gradient being greater the lower the ambient temperature.

2. Singing is preceded by a warm-up period during which normally antagonistic forewing muscles contract synchronously. The result is heat production and a rising thoracic temperature (1.5° C/min) with little overt movement.

3. The thoracic temperature at the onset of singing averages 33.5° C at ambient temperatures at 23.5°–29° C.

4. The rate of heat loss at the cessation of singing indicates that the animal must produce 0.5 cal/min (3.6 cal/min/g thorax weight) to maintain a 15° C temperature gradient.

5. This insect is immobilized by cooling to 11° C, but regains mobility when rewarmed to 21.5° C. It is reversibly immobilized at thoracic temperatures above 43.9° C.

6. *N. robustus* basks readily, but moves to shade at body temperatures of 37.6° C. Shuttling between sunlight and shade would permit this insect to regulate

its body temperature to a high but restricted temperature range during daylight hours.

LITERATURE CITED

Alexander, E. D. 1956. A Comparative Study of Sound Production in Insects, With Special Reference to the Singing Orthoptera and Cicadidae of the Eastern United States. *Ph.D. thesis, Ohio State University*, 528 pp.

Church, N. S., 1960. Heat loss and body temperature of flying insects Part I and II. *J. Exp. Biol.*, **37**: 171–212.

Dumortier, B., 1964. Morphology of sound emission apparatus in arthropoda, pp. 277–345. *In:* R. G. Busnel, Ed., *Acoustic Behaviour of Animals.* Elsevier Publishing Co., Amsterdam.

Esch, H., 1968. La thermoregulation chez l'abeille, pp. 215–223. *In: Traité de Biologie de l'abeille, Volume I.* Masson et Cie, Paris.

Heath, J. E., 1967. Temperature responses of the periodical "17-year" cicada, *Magicicada cassini* (Homoptera, Cicadidae) *Amer. Midland Natur.*, **77**: 64–76.

Heath, J. E., 1968a. Insect temperature regulation, pp. 221–222. *In: McGraw-Hill Yearbook of Science and Technology.* McGraw-Hill, New York.

Heath, J. E., 1968b. The origins of thermoregulation, pp. 259–278. *In:* E. T. Drake, Ed., *Evolution and Environment.* Yale Univ. Press., New Haven, Connecticut.

Heath, J. E., and P. A. Adams, 1967. Regulation of heat production by large moths. *J. Exp. Biol.*, **47**: 21–23.

Heath, J. E., and P. A. Adams, 1969. Temperature regulation and heat production in insects, pp. 275–293. *In:* G. A. Kerkut, Ed., *Experiments in Comparative Biochemistry and Physiology, Volume II.* Academic Press, London.

Josephson, R. K., and H. Y. Elder, 1968. Rapidly contracting muscles used in sound production by a katydid. *Biol. Bull.*, **135**: 409.

Kammer, A. E., 1968. Motor patterns during flight and warm-up in Lepidoptera. *J. Exp. Biol.*, **48**: 89–109.

Krogh, A., and E. Zeuthen, 1941. The mechanism of flight preparation in some insects. *J. Exp. Biol.*, **18**: 1–10.

McCrea, M. J., 1969. Temperature regulation and control of warm-up behavior of the sphinx moth, *Manduca sexta. Ph.D. thesis, University of Illinois,* 104 pp.

Neville, A. C., and T. Weis-Fogh, 1963. The effect of temperature on locust flight muscle. *J. Exp. Biol.*, **40**: 111–121.

Pierce, G. W., 1948. *The Songs of Insects.* Harvard University Press, Cambridge, 329 pp.

Prosser, C. L., and F. A. Brown, 1961. *Comparative Animal Physiology,* [2nd. ed.] W. B. Saunders Co., Philadelphia, 688 pp.

Sotavalta, O., 1954. On the thoracic temperature of insects in flight. *Suomalaisen eläin-ja Kasvitieteellisen Seuran Vanamon, Eläintieteellisiä Julkaisuja* (*Annales Zoologici Societatis Zoologicae-Botanicae Fennicae Vanamo*), **16**: 7–22.

Weis-Fogh, T., 1954. Fat consumption and metabolic rate of flying locusts (*Schistocerca gregaria* Forskål). *Phil. Trans. Roy. Soc. London, Series B*, **237**: 1–36.

Reference: *Biol. Bull.*, **138**: 286–305. (June, 1970).

A COMPARATIVE STUDY OF GUT MUCOUS CELLS IN THIRTY-SEVEN SPECIES OF THE CLASS ECHINOIDEA (ECHINODERMATA)

NICHOLAS D. HOLLAND AND MICHAEL T. GHISELIN

Division of Marine Biology, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92037, and Department of Zoology, University of California, Berkeley, California 94720

The gut of echinoids is lined by an epithelium which includes some mucous cells; the structure and function of echinoid guts in general has recently been reviewed by Anderson (1966). The term *mucous cell* is restricted in the present paper to denote only those epithelial cells containing a conspicuous accumulation of mucus intracellularly. In addition, the lining epithelium of echinoid guts may include the following two kinds of cells associated with mucus, yet not considered here to be mucous cells: (1) mucus-containing coelomocytes wandering in the gut wall and (2) epithelial cells lacking intracellular mucus, but having a thin glycocalyx of mucus associated with the distal cell membrane. Mucous cells in echinoid guts were first mentioned explicitly by Koehler in 1883; unfortunately, his report is hard to evaluate, because it came long before the advent of histochemistry. Since the development of histochemical methods for mucopolysaccharides, gut mucous cells have been studied in some detail for the following echinoid species: *Echinus esculentus* (Stott, 1955; Buchanan, 1969), *Strongylocentrotus intermedius* (Fuji, 1961), *Strongylocentrotus purpuratus* (Holland and Nimitz, 1964), and *Psammechinus miliaris* (Buchanan, 1969). Less detailed studies have been made of the gut mucous cells in *Diadema antillarum* (Lewis, 1964) and *Dendraster excentricus* (Reisman, 1965).

The species mentioned above represent but a few of the extant orders and families of echinoids. The lack of information about mucous cells in the class Echinoidea as a whole prompted the present investigation of the patterns of distribution and the histochemical properties of mucous cells in the guts of thirty-seven species of echinoids, both regular and irregular. The same set of histochemical methods was applied to each species in order to facilitate comparisons. The results have been interpreted phylogenetically in the context of a widely accepted scheme of macroevolution in the class Echinoidea (Durham and Melville, 1957). The echinoid taxonomy of Mortensen (1928, 1935, 1940, 1943a, 1943b, 1948, 1950, 1951) has been followed here because it is more suitable at the ordinal level than the newer echinoid taxonomy (summarized by McCormick and Moore, 1966) for condensing and summarizing the results of the present investigation. During the course of the present study, some new facts about the gross anatomy of the echinoid siphon unexpectedly came to light and have necessarily been reported here.

To date, the terminology for echinoid gut regions has not been standardized. Therefore, the terms used in this paper must be defined at the outset. In the regular echinoids, the regions of the main course of the digestive tract are the following: (1) the *buccal cavity* begins with the mouth at the center of the oral surface and extends to the level of the nerve ring; (2) the *pharynx* begins at the level of the nerve ring and extends to the aboral surface of the lantern; (3) the *esophagus* begins at the aboral surface of the lantern and extends to the place where the gut diameter suddenly increases, marking the start of the stomach; (4) the *stomach* makes a nearly complete circuit of the body to the beginning of the intestine; (5) the *intestine* doubles back aboral to the stomach and makes a nearly complete circuit of the body to end indistinctly with the rectum; (6) the short *rectum* ascends to the aboral pole and terminates at the anus. In the regular echinoid orders Lepidocentroida, Stirodonta and Camarodonta, a slender tube termed the *siphon* leaves the main course of the gut near the junction of the esophagus and stomach, runs parallel to the stomach and rejoins the main course of the gut near the junction of the stomach and intestine. In the regular echinoid orders Cidaroida (Prouho, 1887) and Aulodonta (as the present investigation brought to light), there is no siphon; in its place, a *siphonal groove* runs the entire length of the stomach.

In irregular echinoids of the order Clypeasteroida, the regions of the main course of the digestive tract are the following: (1) the *buccal cavity* begins with the mouth at the center of the oral surface and extends to the level of the nerve ring; (2) a very short *pharynx* begins at the level of the nerve ring and extends to the aboral surface of the low lantern; (3) a short *esophagus* begins at the aboral surface of the lantern and extends to the place where the *siphon* leaves the main course of the gut and where the gut diameter abruptly increases, marking the start of the stomach; (4) the *stomach* makes half a circuit of the body to a place where the siphon rejoins the main course of the gut, marking the start of the intestine; (5) the *intestine* completes the circuit of the body started by the stomach and then doubles back on itself aborally, making half a circuit of the body to end indistinctly with the short rectum; (6) the *rectum* terminates at the anus on the oral side of the animal in the posterior interambulacrum.

In irregular echinoids of the order Spatangoida, the lantern and pharynx are missing. Thus the regions of the main course of the digestive tract are the following: (1) the *buccal cavity* begins with the mouth displaced anteriorly on the oral surface and extends to the level of the nerve ring; (2) the *esophagus* begins at the level of the nerve ring and extends to the place where the *siphon* leaves the main course of the digestive tract, marking the start of the stomach (for spatangoids, it is useful to create the term *distal esophagus* for a relatively short portion of esophagus immediately preceding the stomach and the term *proximal esophagus* for the remainder of the esophagus); (3) the *stomach* makes a complete circuit of the body to a place where the siphon rejoins the main course of the gut, marking the start of the intestine (shortly after it arises from the esophagus, the stomach gives off a conspicuous *caecum*); (4) the *intestine* continues briefly the circuit of the body started by the stomach, doubles back aboral to the stomach, makes a nearly complete circuit of the body, doubles back aboral to itself and runs posteriorly to end indistinctly with a short rectum; (5) the *rectum* terminates at the anus near the ambitus of the posterior interambulacrum. In a few spatangoid species, superimposed on the general gut plan above, there is a second caecum at the beginning of the rectum; also in a few species there may be a relatively short accessory siphon lying between and parallel to the stomach and the siphon.

With the terminology established above, it is very likely that regular echinoid gut regions are homologous to clypeasteroid gut regions of the same name. Somewhat less secure are the homologies between spatangoid gut regions and gut regions of the same name in non-spatangoid echinoids. In spite of this, it will be assumed in this paper that the names of the gut regions defined here reflect homologies accurately within the class Echinoidea.

Materials and Methods

Table I enumerates the thirty-seven echinoid species investigated and gives the place and depth where each was collected. Whenever possible, at least four specimens of each species were studied. However, only two specimens could be obtained for *Diadema mexicanum, Echinus melo, Maretia planulata, Lovenia subcarinata* and *Brissopsis* sp.; only one specimen could be obtained for *Echinothrix calamaris* and *Centrostephanus longispinus.* Most species were fixed in sea water-Bouin's fluid, but the following species were fixed in 10% formalin: *Sperosoma crassispinum, Astropyga pulvinata, Clypeaster reticulatus, Urechinus narescianus, Maretia planulata, Lovenia subcarinata* and *Brissopsis* sp. Presumably these differences in fixation were not important, since mucous cells of *Strongylocentrotus purpuratus* reacted similarly to the histochemical tests used here, whether fixed in sea water-Bouin's fluid (Holland and Nimitz, 1964) or in 10% formalin (Holland, unpublished observation).

For as many species as possible, an entire, small individual was embedded without dissection to permit serial reconstruction of the whole gut. Otherwise, during dehydration, the gut from each larger individual was removed and dissected into the following regions: (1) pharynx (except in spatangoids), (2) esophagus, (3) stomach including siphon or siphonal groove, (4) intestine and (5) rectum. The present study did not include mucous cells present in any part of the buccal cavities of echinoids. In general, the histological procedures described on pp. 283–284 of Holland and Nimitz (1964) were followed; sand grains present in the gut of some irregular echinoids were simply shaken out vigorously after the gut segments had been dehydrated as far as 95% ethanol.

For each of the thirty-seven species, sections of each gut region were stained by the following procedures:

(1) The periodic acid-Schiff (PAS) method for aqueous solutions was used according to the instructions of Humason (1962). To remove any glycogen that might have survived histological processing, sections were treated with diastase before staining (Pearse, 1960).

(2) Alcian blue at pH 3 (Al Bl) was used according to directions of Humason (1962), omitting the counterstain. Some sections were stained sequentially with alcian blue and then PAS.

(3) Azure A (Az A) was used according to instructions of Casselman (1959). Some sections were stained sequentially with PAS and then Azure A.

The incorporation of S^{35}-sulfate into gut mucous cells was investigated autoradiographically in seven of the species listed in Table I. A sea water solution of $Na_2S^{35}O_4$ (Amersham, carrier free) was injected intracoelomically into two

Table I

Echinoid species studied histochemically

Taxa	Place of collection	Depth (m)
Class Echinoidea, Subclass Regularia		
Order Cidaroidea		
Family Cidaridae		
Stylocidaris affinis	Gulf of Naples, Italy	70
Eucidaris thouarsii	Espiritu Santo Is., Mexico	1
Eucidaris tribuloides	Discovery Bay, Jamaica	2
Prionocidaris baculosa	Wadi el Dom, Egypt, UAR	3
Order Aulodonta		
Family Diadematidae		
Astropyga pulvinata	Acapulco, Mexico	20
Diadema setosum	Wadi el Dom, Egypt, UAR	1
Diadema antillarum	Discovery Bay, Jamaica	2
Diadema mexicanum	Cabo San Lucas, Mexico	2
Echinothrix calamaris	Quseir, Egypt, UAR	3
Centrostephanus longispinus	Gulf of Naples, Italy	40
Centrostephanus coronatus	Sta. Catalina Is., Cal., U. S. A.	15
Order Lepidocentroida		
Family Echinothuriidae		
Sperosoma crassispinum	70 mi. off Cabo San Quintin, Mexico	1600 and 2100
Order Stirodonta		
Family Arbaciidae		
Arbacia lixula	Gulf of Naples, Italy	1
Arbacia punctulata	Woods Hole, Mass., U. S. A.	supply dept.
Arbacia stellata	Guaymas, Mexico	1
Order Camarodonta		
Family Temnopleuridae		
Genocidaris maculata	Gulf of Naples, Italy	70
Family Toxopneustidae		
Lytechinus variegatus	Discovery Bay, Jamaica	2
Lytechinus anamesis	La Jolla, Cal., U. S. A.	10
Tripneustes ventricosus	Discovery Bay, Jamaica	2
Tripneustes gratilla	Safaga, Egypt, UAR	2
Sphaerechinus granularis	Gulf of Naples, Italy	10
Family Echinidae		
Echinus melo	Gulf of Naples, Italy	25
Psammechinus microtuberculatus	Gulf of Naples, Italy	8
Paracentrotus lividus	Gulf of Naples, Italy	2
Family Strongylocentrotidae		
Strongylocentrotus franciscanus	La Jolla, Cal., U. S. A.	15

TABLE I—(*Continued*)

Taxa	Place of collection	Depth (m)
Family Echinometridae		
Echinometra lucunter	Discovery Bay, Jamaica	2
Echinometra vanbrunti	Espiritu Santo Is., Mexico	2
Echinometra mathaei	Wadi el Dom, Egypt, UAR	2
Heterocentrotus mammillatus	Quseir, Egypt, UAR	3
Class Echinoidea, Subclass Irregularia		
Order Clypeasteroida		
Family Clypeasteridae		
Clypeaster reticulatus	Nero Bank (Midpacific)	70
Family Scutellidae		
Dendraster excentricus	Newport Beach, Cal., U. S. A.	7
Mellita longifissa	San Felipe, Mexico	1
Encope grandis	San Felipe, Mexico	1
Order Spatangoida		
Suborder Meridosternata		
Family Urechinidae		
Urechinus narescianus	70 mi. off Cabo San Quintin, Mexico	1600
Suborder Amphisternata		
Family Spatangidae		
Maretia planulata	Nha Trang Bay, Viet Nam	26
Family Loveniidae		
Lovenia subcarinata	Ko Samui, Gulf of Siam	23
Family Brissidae		
Brissopsis sp.	Guaymas, Mexico	3

small individuals of each of the following species: *Stylocidaris affinis, Arbacia lixula, Centrostephanus coronatus, Genocidaris maculata, Sphaerechinus granularis, Psammechinus microtuberculatus,* and *Paracentrotus lividus.* The dose for each individual was 10 microcuries of S^{35} per gram of fresh weight. For each species, one animal was killed one hour after injection and the other was killed 24 hours after injection. After histological processing, the specimens were sectioned and covered with Kodak AR-10 stripping film for autoradiography. After exposure for three weeks, the autoradiograms were developed and the sections were stained through the emulsion with azure A in 30% ethanol.

RESULTS

The histology of all gut regions of the thirty-seven echinoid species studied conformed to the pattern elucidated in earlier investigations of echinoid guts. The inner epithelium was composed of columnar epithelial cells, some of which were mucous cells. Peripheral to the inner epithelial layer was a middle layer consisting of connective tissue, muscles and a nerve plexus. Peripheral to the connective tissue-muscle layer was a thin visceral peritoneum (also termed the coelomic epithelium). For the purposes of the present investigation, the middle layer and the visceral peritoneum need not be considered further—except in the case of the distal esophagus of amphisternate spatangoids.

Within each mucous cell of the lining epithelium, histochemically demonstrable mucus was always concentrated in the apical (also called luminal or distal) region of the cell. This apical, mucus filled cell region occupied between 10% and 90% of the total mucous cell length. For the class Echinoidea as a whole, it was convenient to divide the mucous cells of the gut into three categories based on the relation between the mucous cell and the neighboring epithelial cells. (1) *Aggregated mucous cells,* wherever found in the gut, constituted almost pure cell populations with the interspersion of few other epithelial cell types. The apical, mucus filled portion of aggregated mucous cells always reached the lumen of the gut. A population of aggregated mucous cells thus existed as a secretory epithelial sheet, considered by Maximow and Bloom (1957) to be the simplest kind of multicellular gland. (2) *Solitary distal mucous cells* were relatively sparsely distributed among other epithelial cell types and the apical, mucus filled portion always reached the gut lumen. Even those cells that had a basal, mucus swollen region were considered solitary distal mucous cells if the basal mucus communicated with the lumen via a narrow, mucus filled neck. (3) *Solitary deep mucous cells* were relatively sparsely distributed in the epithelium and were shorter than the surrounding epithelial cells; thus the distal, mucus filled region did not reach the gut lumen. Solitary mucous cells (distal or deep) are unicellular glands in the terminology of Maximow and Bloom (1957).

In the mucous cells of echinoid guts, the following three histochemical classes of mucus could be distinguished: (1) sulfated acid mucus, (2) nonsulfated acid mucus and (3) neutral mucus. Each class of mucus was defined by a set of histochemical criteria (Table II) taken from Spicer's 1963 classification of mammalian mucus. The criteria of Spicer have been modified in one respect; orthochromatic (*i.e.*, blue) staining with azure A and the fixation of S^{35}-sulfate, which were never correlated in mammalian mucus, were always correlated in echinoid gut mucus. Therefore, orthochromatic staining with azure A, a property of nonsulfated acid mucus in mammals, was considered to be a property of sulfated acid mucus in the present investigation.

At the gross anatomical level, Figure 1 summarizes the patterns of distribution shown by mucous cells along the course of the digestive tract in echinoids. Among the regular echinoids, mucous cells are most widely distributed in cidaroids, occurring in all gut regions except the siphonal groove. Some aulodont species have

TABLE II

Histochemical criteria defining classes of mucus

Type of mucus	PAS*	Al Bl	Az A	S^{35}
Sulfated acid mucus	− to +	− to +	+**	+
Nonsulfated acid mucus	− to +	+	−	−
Neutral mucus	+	−	−	−

* With prior diastase digestion.

** Orthochromasia or metachromasia.

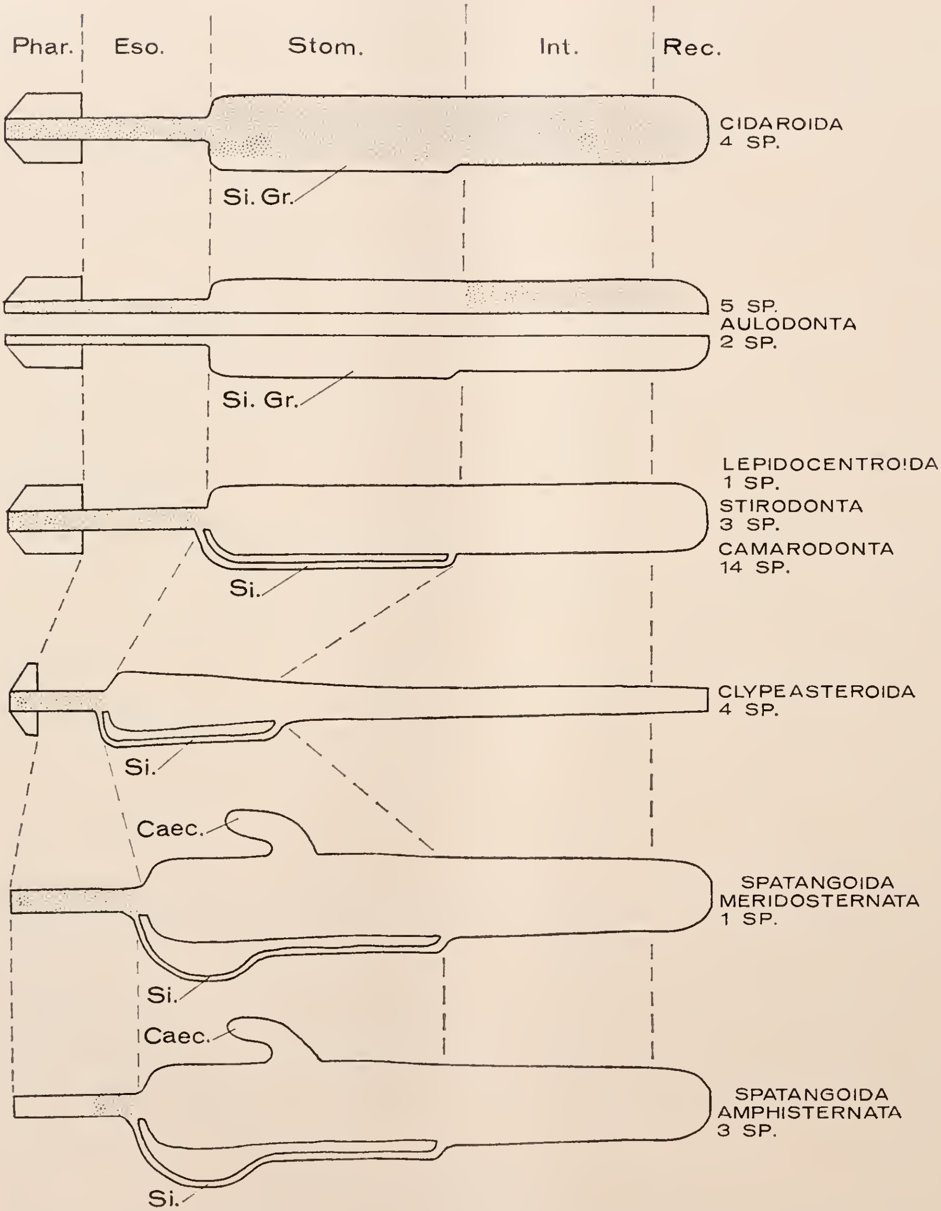


FIGURE 1. A summary, at the gross anatomical level, of the patterns of distribution shown by mucous cells along the course of the gut in echinoids. The presence of mucous cells in a gut region is indicated by stippling. For simplicity, the six digestive tracts have been drawn straightened out from the mouth (at the left) to the anus (at the right). The names of the main gut regions appear at the top of the figure and are abbreviated as follows: Phar., pharynx; Eso., esophagus; Stom., stomach; Int., intestine; Rec., rectum. Also labeled are the siphonal groove (Si. Gr.), siphon (Si.) and caecum (Caec.) wherever they appear in the figure. For orientation, the lantern of Aristotle has been drawn around the pharynx. The echinoid taxa described by each of the six gut diagrams are given at the right side of the figure; the aulodont diagram is split to accommodate the two different distributional patterns in that order.

mucous cells distributed in all gut regions except the stomach and siphonal groove, while other aulodont species have mucous cells distributed in the pharynx and esophagus, but not in any gut regions following the junction of the esophagus and stomach. Like these latter aulodonts, the lepidocentroids, stirodonts and camarodonts have mucous cells limited to the pharynx and esophagus. In irregular echinoids of the order Clypeasteroida, mucous cells are distributed in the pharynx and esophagus but are absent from all gut regions following the junction of the esophagus and stomach. In irregular echinoids of the order Spatangoida, the pharynx with its mucous cells has disappeared, leaving the esophagus as the only gut region containing mucous cells. The esophageal mucous cells of spatangoids are distributed along the entire length of the esophagus in the suborder Meridosternata but are restricted to the distal part of the esophagus in the suborder Amphisternata. The detailed histochemistry of the mucous cells of echinoid guts will be described below, group by group, in the same taxonomic sequence already presented in Table I and Figure 1.

Subclass Regularia

Within each regular echinoid order except the Aulodonta, the detailed distributional patterns and histochemistry of the mucous cells were quite consistent from species to species. Therefore, in Figure 2, one set of diagrams was sufficient to describe all species studied within each order—excepting the order Aulodonta, for which two diagrams were necessary. For the interpretation of the cross sectional diagrams of Figure 2, it is necessary to review, briefly, selected anatomical features of each gut region. The most striking gross anatomical features shown in the pharyngeal cross sections are the five radial grooves, where the lining epithelial cells are shorter than in the five interradial ridges; each groove and each ridge runs uninterrupted throughout the length of the pharynx and is conspicuous in gross dissection. The esophageal cross sections are characterized by deep, narrow esophageal grooves (actually more numerous than depicted) where the lining epithelial cells are shorter than in the intervening regions of thick epithelium. The longitudinal esophageal grooves shown in Figure 2 are repeatedly intersected by radial esophageal grooves (not shown), dividing the regions of thick epithelium into approximately rectangular patches; this gives to a surface view of inverted esophagus the appearance of a shucked ear of maize. Superimposed on this basic esophageal morphology of regular echinoids are the intraepithelial glands peculiar to some species of the class Aulodonta; these glands will be described further below. Stomach cross sections often have scattered, shallow notches in the luminal edge of the lining epithelium; these notches correspond to long, shallow creases that appear to be relatively permanent morphological features observable with difficulty in gross dissections. Not to be confused with these shallow notches is the much deeper and wider siphonal groove running the length of the stomach in cidaroids and aulodonts. Intestine and rectum cross sections are characterized by luminal notches comparable to those of the stomach.

Order Cidaroidea (Stylocidaris affinis, Eucidaris thouarsii, Eucidaris tribuloides *and* Prionocidaris baculosa)

In the pharynx, as shown in Figure 2, the epithelium lining the radial grooves consisted almost entirely of aggregated mucous cells containing sulfated acid mucus; in the interradial ridges were scattered solitary distal mucous cells, some containing sulfated acid mucus, some containing nonsulfated acid mucus and some containing neutral mucus. In the esophagus, most of the cells of the lining epithelium were aggregated mucous cells containing sulfated acid mucus. Scattered in the regions of thick esophageal epithelium were a few solitary deep mucous cells, some containing sulfated acid mucus, some containing nonsulfated acid mucus and some containing neutral mucus. In the stomach, aggregated mucous cells containing sulfated acid mucus were always associated with the luminal notches but usually occurred in isolated patches elsewhere; the epithelium lining of the siphonal groove was free of mucous cells. In the intestine and rectum, virtually the entire epithelium consisted of aggregated mucous cells containing sulfated acid mucus.

Order Aulodonta, group one (Astropyga pulvinata, Diadema setosum, Diadema antillarum, Diadema mexicanum *and* Echinothrix calamaris)

In the pharynx, as shown in Figure 2, solitary distal mucous cells containing sulfated acid mucus were scattered in the epithelium of the radial grooves; possibly similar mucous cells present in the interradial ridges could be demonstrated faintly only by prolonged staining in azure A and have not been indicated in Figure 2. Also present in the interradial pharyngeal ridges of the pharynx were solitary distal mucous cells containing either nonsulfated acid mucus or neutral mucus. In the esophagus, much of the epithelium consisted of aggregated mucous cells containing sulfated acid mucus. Scattered in the regions of thick esophageal epithelium were solitary deep mucous cells that contained sulfated acid mucus, nonsulfated acid mucus or neutral mucus. Unique to the present group of aulodont species were intraepithelial glands (in the terminology of Maximow and Bloom, 1957). These intraepithelial glands were first described for *Diadema antillarum* by Lewis in 1964; each gland was a small accumulation of aggregated mucous cells lying wholly within the epithelium and surrounding a small gland lumen. The intraepithelial gland lumen communicated with the esophageal lumen via an opening either into an esophageal groove or in a region of thick esophageal epithelium; the mucous cells of these glands contained sulfated acid mucus or nonsulfated acid mucus. Scattered in the regions of thick esophageal epithelium were solitary deep mucous cells, some containing sulfated acid mucus, some containing nonsulfated acid mucus and some containing neutral mucus. Although mucous cells were absent from the stomach and the siphonal groove, solitary distal mucous cells containing sulfated acid mucus were present in the intestine and rectum; these cells were quite sparse in some individual urchins and relatively abundant in others.

Order Aulodonta, group two (Centrostephanus longispinus *and* Centrostephanus coronatus)

In the pharynx, as shown in Figure 2, the epithelium lining the radial grooves consisted almost entirely of aggregated mucous cells containing sulfated acid mucus. In the interradial ridges of the pharynx were scattered solitary distal mucous cells containing sulfated acid mucus, nonsulfated acid mucus or neutral mucus. In the esophagus, much of the epithelium consisted of aggregated mucous cells containing sulfated acid mucus; scattered in the regions of thick esophageal epithelium were

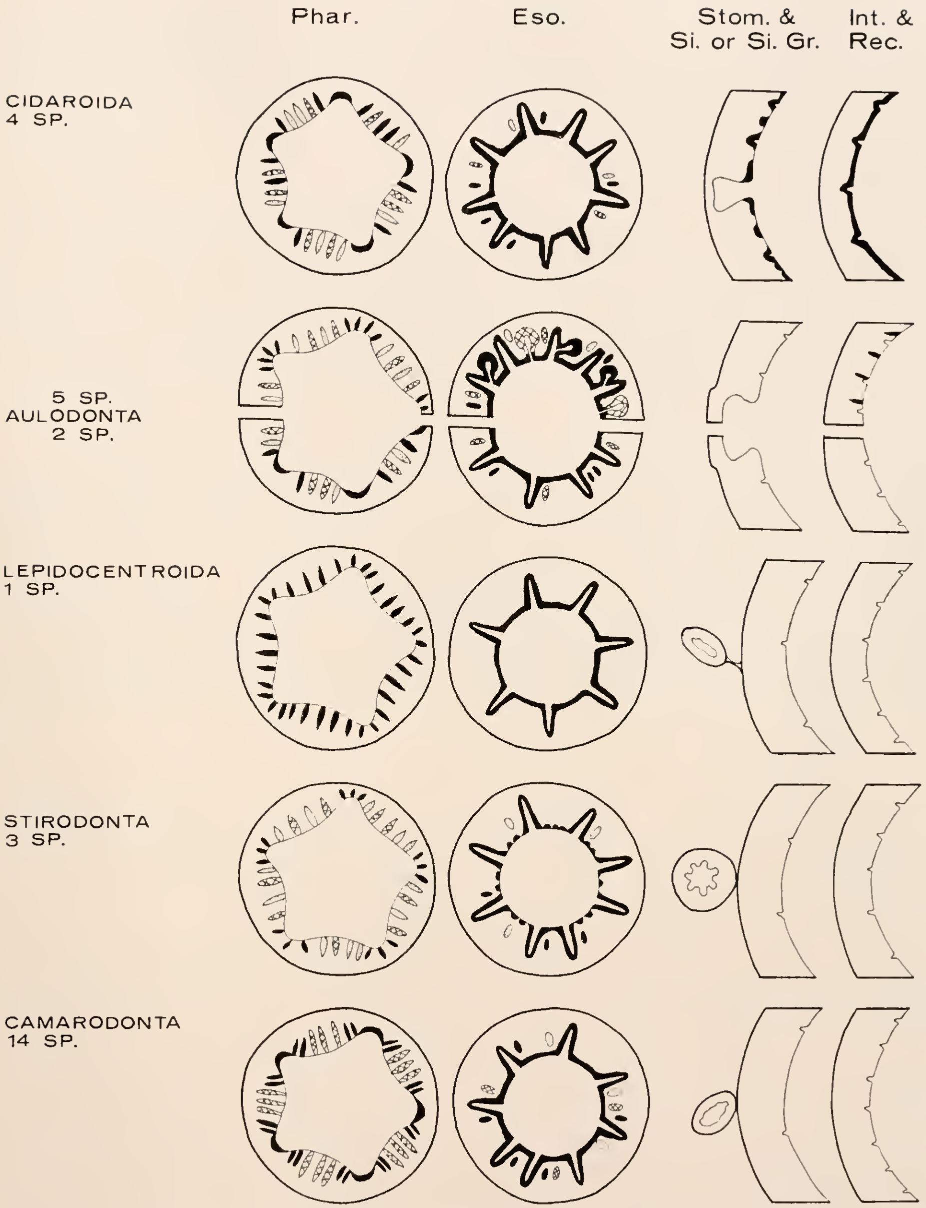


FIGURE 2. The detailed distributional patterns and histochemistry of the gut mucous cells in regular echinoids. For each of the regular echinoid orders listed at the left side of the figure, the gut is represented diagrammatically by a set of four cross sectional diagrams, the names of which are given at the top of the figure (abbreviations are as in Fig. 1). The cross sectional diagrams are complete for the pharynx and esophagus, but are partial for other gut regions. The set of aulodont diagrams is split to accommodate the two different distributional patterns in that order. In the diagrams, sulfated acid mucus is represented by solid black, nonsulfated acid mucus by cross hatching and neutral mucus by stippling.

solitary deep mucous cells, containing either sulfated acid mucus or nonsulfated acid mucus. There were no intraepithelial glands in the esophagus of these two aulodont species. The stomach, siphonal groove, intestine and rectum contained no mucous cells whatever.

Order Lepidocentroida (Sperosoma crassispinum)

In the pharynx, as shown in Figure 2, solitary distal mucous cells containing sulfated acid mucus were scattered in the epithelium lining the radial grooves and the interradial ridges. In the esophagus, virtually the entire lining epithelium consisted of aggregated mucous cells containing sulfated acid mucus. The stomach, siphon, intestine and rectum were free of epithelial mucous cells, but contained some conspicuous wandering coelomocytes filled with sulfated acid mucus.

Order Stirodonta (Arbacia lixula, Arbacia punctulata *and* Arbacia stellata)

In the pharynx, as shown in Figure 2, solitary distal mucous cells containing sulfated acid mucus were sparsely scattered in the epithelium of the radial grooves; solitary distal mucous cells containing either nonsulfated acid mucus or neutral mucus were present in the interradial ridges. In the esophagus, aggregated mucous cells containing sulfated acid mucus were always present in the epithelium of the esophageal grooves but frequently had a relatively patchy distribution in the regions of thick epithelium; scattered in the regions of thick epithelium were solitary deep mucous cells containing either sulfated acid mucus or neutral mucus. The stomach, siphon, intestine and rectum contained no mucous cells whatever.

Order Camarodonta (Genocidaris maculata, Lytechinus variegatus, Lytechinus anamesis, Tripneustes ventricosus, Tripneustes gratilla, Sphaerechinus granularis, Echinus melo, Psammechinus microtubercultaus, Paracentrotus lividus, Strongylocentrotus franciscanus, Echinometra lucunter, Echinometra vanbrunti, Echinometra mathaei *and* Heterocentrotus mammillatus)

In the pharynx, as shown in Figure 2, the epithelium lining the radial grooves consisted almost entirely of aggregated mucous cells containing sulfated acid mucus. In the interradial ridges of the pharynx were scattered solitary distal mucous cells, some containing sulfated acid mucus and some containing nonsulfated acid mucus; the former were most abundant near the radial grooves, while the latter were most abundant interradially. In the esophagus, most of the epithelium consisted of aggregated mucous cells containing sulfated acid mucus. Scattered in the regions of thick esophageal epithelium were solitary deep mucous cells containing sulfated acid mucus, nonsulfated acid mucus or neutral mucus (no solitary deep mucous cells whatever were seen in any specimen of *Lytechinus anamesis*, *Sphaerechinus granularis*, or *Echinus melo*). There were no mucous cells of any kind in the stomach, siphon, intestine and rectum. The results for these 14 camarodont echinoids are in general agreement with previous work on three other camarodont species; these studies were by Holland and Nimitz (1964) on *Strongylocentrotus purpuratus* and by Buchanan (1969) on *Echinus esculentus* and *Psammechinus miliaris*.

Uptake of S^{35}-sulfate and subsequent fate of S^{35}-labeled mucus in gut mucous cells of regular echinoids

In the seven regular echinoid species studied autoradiographically, S^{35}-sulfate was incorporated only into those mucous cells that contained sulfated acid mucus. One pattern of incorporation was seen in the solitary distal mucous cells and a second pattern of incorporation was seen in the aggregated mucous cells and in solitary deep mucous cells containing sulfated acid mucus. In the solitary distal mucous cells, the S^{35}-sulfate was first incorporated into the most basal part of the mucus-filled region of the cell; presumably, the S^{35}-sulfate had been incorporated into newly synthesized sulfated acid mucus that was stainable histochemically. By 24 hours after administration of S^{35}-sulfate, the initially sulfated mucus had spread distally throughout the mucus-filled region of the cell. By contrast, in aggregated mucous cells and in solitary deep mucous cells containing sulfated acid mucus, the S^{35}-sulfate was first incorporated in a cell region where no mucus was demonstrable histochemically (approximately in the middle third of the cell). By 24 hours after administration of S^{35}-sulfate, the initially sulfated material had migrated distally into the mucus-filled region of the cell. Such results, previously reported for the aggregated mucous cells in the esophagus of *Strongylocentrotus purpuratus* (Holland and Nimitz, 1964), were presumably due to the following sequence of events: (1) incorporation of S^{35}-sulfate into newly synthesized sulfated acid mucus in the middle third of the cell, where the sulfate groups of the young mucus were masked, perhaps by combination with protein; (2) distal migration of the radioactive mucus in its masked state; and finally (3) an unmasking of the sulfate groups as the radioactive mucus reaches the mucus-filled, distal end of the cell.

Subclass Irregularia

Within the irregular echinoid order Clypeasteroida, the detailed distributional patterns and histochemistry of the gut mucous cells were quite consistent from species to species. Therefore, in Figure 3, one set of diagrams was sufficient to describe all clypeasteroid species studied. By contrast, within the irregular echinoid order Spatangoida, there were two different distributional patterns of the gut mucous cells, one for the suborder Meridosternata and the other for the suborder Amphisternata. Therefore, in Figure 3, two sets of diagrams were needed to describe all spatangoid species studied.

Order Clypeasteroida (Clypeaster reticulatus, Dendraster excentricus, Mellita longifissa *and* Encope grandis)

The clypeasteroid pharynx (Figure 3), like its longer counterpart in regular echinoids, had five radial grooves and five interradial ridges. In the epithelium of the grooves and ridges were scattered a few solitary distal mucous cells, some containing nonsulfated acid mucus and some containing neutral mucus; these latter cells were first described for *Dendraster excentricus* by Reisman in 1965. The clypeasteroid esophagus differed from its counterpart in regular echinoids in having far fewer longitudinal grooves and no radial grooves. In the esophagus of

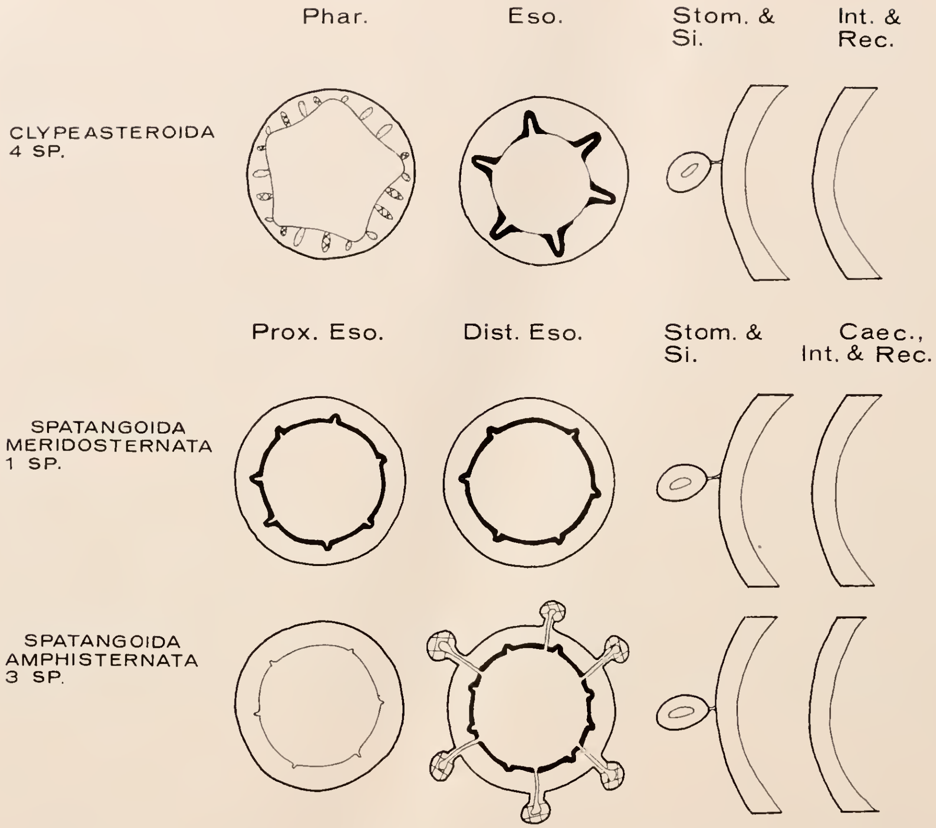


FIGURE 3. The detailed distributional patterns and histochemistry of the gut mucous cells in irregular echinoids. For the order Clypeasteroida, the gut is represented diagrammatically by a set of four cross sectional diagrams, complete for the pharynx and esophagus, but partial for other gut regions; the names of the gut regions are abbreviated as in Figure 1. The order Spatangoida is represented by two sets of cross sectional diagrams, one for the suborder Meridosternata and one for the suborder Amphisternata. For the order Spatangoida, which lacks a pharynx, two complete cross sectional diagrams are presented for the esophagus (Prox. Eso., proximal esophagus and Dist. Eso., distal esophagus); other spatangoid gut regions are represented by partial cross sectional diagrams, the names of which are abbreviated as in Figure 1. In the distal esophagus of amphisternate spatangoids, the simple acinous glands are actually embedded in a thick middle layer of the gut wall that has not been included in any of the diagrams. In the diagrams, sulfated acid mucus is represented by solid black, nonsulfated acid mucus by cross hatching and neutral mucus by stippling.

the family Clypeasteridae, virtually the entire epithelium was composed of aggregated mucous cells containing sulfated acid mucus. In the esophagus of the family Scutellidae, aggregated mucous cells containing sulfated acid mucus lined some or all of the longitudinal esophageal grooves but were absent from the regions of thick esophageal epithelium; Reisman (1965) termed these cells esophageal goblet cells in *Dendraster excentricus*. There were no mucous cells of any kind in the stomach, siphon, intestine and rectum of clypeasteroids. The mucous cells that

Reisman (1965) reported in the siphon of *Dendraster excentricus* appear to have been coelomocytes wandering in the lining epithelium.

Order Spatangoida, Suborder Meridosternata (Urechinus narescianus)

In *Urechinus,* virtually the entire epithelium lining the esophagus (comprised by the proximal esophagus plus the distal esophagus) was composed of aggregated mucous cells containing sulfated acid mucus (Fig. 3). The stomach, siphon, caecum, intestine and rectum were completely free of mucous cells.

Order Spatangoida, Suborder Amphisternata (Maretia planulata, Lovenia subcarinata *and* Brissopsis *sp.*)

Unlike the meridosternate spatangoids, the amphisternate spatangoids has no mucous cells in the anterior three-fourths of the esophagus (the proximal esophagus). In the suborder Amphisternata, aggregated mucous cells containing sulfated acid mucus were limited to the posterior one-fourth of the esophagus (the distal esophagus) where they made up virtually the entire lining epithelium (Fig. 3). Also peculiar to the distal esophagus were simple acinous glands (in the terminology of Maximow and Bloom, 1957) that were invaginated into the middle layer of the gut wall and communicated with the gut lumen via a short duct. These simple acinous glands were first seen in the distal esophagus of *Spatangus purpureus* by Koehler (1883), who did not speculate on what their secretory product might be. The present investigation revealed that most of the simple acinous glands of *Lovenia subcarinata* contained nonsulfated acid mucus. However, only a minority of the simple acinous glands of *Maretia planulata* and *Brissopsis* sp. contained nonsulfated acid mucus, while the remainder of the glands did not contain any mucus. The stomach, siphon, caecum, intestine and rectum were completely free of mucous cells.

Discussion

In the present paper, it has been assumed that histochemical results based on a few individuals of one echinoid species are valid for all individuals of that species. This assumption is supported by a study of over seventy specimens of *Strongylocentrotus purpuratus* (Holland, unpublished observations) that demonstrated a remarkable consistency in the distribution and histochemical properties of the mucous cells from animal to animal. The results were independent of each animal's sex, recent nutritional history and postlarval size. To date there has been no study of mucous cells in the gut of larval echinoids.

From the histological and autoradiographic evidence, there is no doubt that the mucus of echinoid gut mucous cells is destined for ultimate export into the gut lumen. Several functions of gut mucus in echinoid digestive biology have been suggested, but there may well be additional functions unsuspected at present. Holland and Nimitz (1964) speculated that lubrication by gut mucus might protect the relatively narrow pharynx and esophagus during the passage of large, sharp pieces of food; however, such an argument does not explain the presence of mucous cells in relatively spacious gut regions of cidaroids and many aulodonts.

Several workers (reviewed on page 52 of Buchanan, 1969) have reported the formation of a thin mucous sheath around food particles taken in by regular echinoids. In the opinion of Buchanan (1969), the mucous sheath is formed only in the buccal cavity and pharynx and thereafter remains intact around the food particle during its passage through the entire course of the gut. In the case of larger food particles trapped and passed to the mouth by spines and podia, the role of the mucous sheath in digestive biology remains enigmatic. In the case of small nutrient particles in suspension in the sea water, the mucus of the buccal cavity of regular echinoids can function in food trapping. Leighton (1968) found that *Artemia* eggs were sucked directly into the mouth of *Strongylocentrotus purpuratus* by a repeated opening and closing of the jaws. After five or ten *Artemia* eggs had stuck to the mucus lining of the buccal cavity, they were swallowed encapsulated in the mucus. In irregular echinoids, food trapping by mucus is of paramount importance (Chesher, 1963; Chia, 1969); however, the mucus used for this purpose is produced not by gut mucous cells but rather by epidermal mucous cells (Nichols, 1959; Chia, 1969).

The present investigation reveals a series of evolutionary trends which are readily reconciled with current morphological and paleontological interpretations of echinoid phylogeny. The Echinoidea in general have gradually (1) lost the mucous cells in the stomach, intestine and rectum; (2) replaced the siphonal groove with a siphon; and (3) reduced somewhat the diversity of histological types of mucus in the pharynx and esophagus. In addition, various taxa have become specialized in characteristic ways, so that each tends to have a pattern of mucous cell types peculiar to itself. On theoretical grounds (Bock, 1959), it is reasonable to expect that those trends which evidently reflect general tendencies having distinct functional advantages should be polyphyletic and irreversible. Thus, a closed siphon is clearly more efficient than an open groove for shunting water around the food mass in the lumen of the stomach and is likely to evolve frequently and not be lost. On the other hand, specializations may lead to some convergence, but, even so, unrelated groups should on the whole differ in at least some details. Such is exactly what we find in echinoid guts when we review the reconstructed history presented by Durham and Melville (1957) and updated somewhat by Durham (1966a).

This evolutionary history of the class Echinoidea, if considered only in its broadest aspects (as stated on pages 247 and 255 of Durham and Melville, 1957), may be discussed in Mortensen's taxonomic terms as follows. Late Palaeozoic cidaroids gave rise to early aulodont stock and also continued the cidaroid line leading to modern cidaroids. From the early aulodont stock, soon after its establishment, the following three different evolutionary lines emerged: the first line led to modern aulodonts, the second to modern lepidocentroids and the third to the stock that subsequently split into lines leading to modern stirodonts, modern camarodonts, modern clypeasteroids and modern spatangoids.

In tracing the probable evolutionary history of the echinoid gut from the Palaeozoic cidaroids to extant echinoids, one can start by hypothesizing that gut features of Palaeozoic cidaroids were fairly close to those of extant cidaroids. Thus, Palaeozoic cidaroids, which probably had the same feeding habits as modern cidaroids (Durham, 1966a) are assumed to have had a siphonal groove along the stomach and a wide-spread distribution of mucous cells in all major gut regions from mouth to anus. In the late Palaeozoic, these primitive echinoid gut features carried over from cidaroids into the earliest aulodont stock. In the evolutionary line leading from early aulodonts to modern aulodonts, the primitive gut features (siphonal groove and wide-spread distribution of gut mucous cells) tended to persist. In one evolutionary line, leading from early aulodonts to modern lepidocentroids, the siphoal groove pinched off from the stomach to form a siphon, and all mucous cells were lost from the stomach, intestine and rectum. In an independent lineage leading from the early aulodonts to the stock that later split into modern stirodonts, camarodonts and irregular echinoids, a siphon likewise formed from the siphonal groove and mucous cells disappeared from the stomach, intestine, and rectum. Some reduction of mucous cells may be detected within the Aulodonta, and therefore *must* have been polyphyletic. At least two origins of the siphon follow if we accept Durham and Melville's inferred relationships. Thus the resemblance of the lepidocentroid gut (in its mucous cell distribution and its siphon) to the guts of stirodonts, camarodonts and irregular echinoids is a result of convergent evolution and not descent from a common ancestor that (1) had a siphon and (2) had mucous cells limited to the pharynx and esophagus.

In the present investigation, the regular echinoid order Camarodonta was well sampled. Among fourteen species belonging to five families, there were no detectable differences in the detailed distribution and histochmistry of the gut mucous cells. In the opinion of Durham and Melville (1957), the camarodont families Temnopleuridae and Toxopneustidae have been separate from the families Echinidae, Strongylocentrotidae and Echinometridae since the late Triassic, about 200 million years ago; presumably, the gut mucous cells have remained unchanged in their distribution and histochemistry at least since the two groups of camarodont families arose from a common ancestral form. This histochemical conservatism of mucous cells does not necessarily reflect a complete lack of change in the genes influencing mucus molecules. Mucus molecules are biochemically complicated, usually having both polysaccharide and protein components synthesized and assembled in a complex multistep process. Most or all of the steps in this process are mediated by enzymes. Therefore, a genetic change (1) could directly alter a structural protein moiety of the mucus molecule or (2) could, by effectively altering an enzyme, indirectly affect the structure of a polysaccaharide moiety or the assembly of the entire mucus molecule. In a mucus molecule, many biochemical changes could occur in the protein moiety (*e.g.,* alterations in amino acid sequences) and in the polysaccharide moiety, *e.g.* (alterations in chain length) without producing any detectable histochemical change. Even if considerable genotypic modification did occur, stabilizing selection ought to conserve the phenotypic properties of the mucus, so long as selection pressures do not change.

The irregular echinoids are now thought to have arisen twice from regular echinoids (Durham, 1966b). The evolutionary line leading to the extant order Spatangoida probably arose from very early stirodont stock, and the line leading to the extant order Clypeasteroida probably diverged subsequently from somewhat later stirodonts. In both lineages, irregular echinoids have generally abandoned the epibenthic habitat of their regular echinoid ancestors to live more or less buried in sand or mud. Durham (1966a) has summarized some of the morphological changes that accompanied the invasion of the stubstrate by irregular echinoids. These changes in the clypeasteroids and in the spatangoids have generally paralleled one another. One important change was the tendency of the body of irregular echinoids to become compressed in its oral-aboral axis. This compression has been accompanied by a shortening of the gut regions preceding the junction of the esophagus and stomach. In the clypeasteroids, both a short pharynx and a short esophagus are present, while, in the spatangoids, the pharynx has disappeared entirely, leaving a relatively short esophagus as the only gut region preceding the stomach. The shortening of the gut regions that contain mucous cells, was, of course, accompanied by the reduction in the number of mucous cells in the gut as a whole. Along with the reduction in gut mucous cells there has been an extensive development of epidermal mucus production in both clypeasteroids (Chia, 1969) and spatangoids (Nichols, 1959; Chesher, 1963). This epidermal mucus is used for food getting by irregular echinoids and large quantities of it doubtlessly enter the mouth mixed with the food. Thus, epidermal mucus entering the digestive tract might well compensate for the reduced amount of gut mucus in irregular echinoids.

One advantage of a good classification system is that it allows us to generalize beyond the limits of our particular observations. By implication, its predictive value enables us to test the validity of the system. The present study illustrates this point quite well: the new histochemical and anatomical data are easily reconciled with taxonomy. Further investigation should provide additional tests of the phylogenetic inferences on which the classifications are based. It is worth considering what we should expect to find when additional echinoid families and orders are studied, because particular kinds of data should be gathered for evaluating the possible hypotheses.

Not all extant families of regular echinoids were covered in the present investigation. In the order Aulodonta, the families Aspidodiadematidae, Micropygidae and Pedinidae were not studied. The Aspidodiadematidae and Micropygidae may be expected to have a siphonal groove rather than a siphon and to have gut mucous cell distributions and histochemistry somewhat like those of the Diadematidae. Mortensen's aulodont family Pedinidae has been allied to the Echinothuriidae by Durham and Melville (1957) and it would, therefore, be extremely interesting to study a pedinid gut to see if a siphon or siphonal groove were present and to see if the mucous cell distribution and histochemistry were of the aulodont type or the lepidocentroid type. In the order Stirodonta, the families Saleniidae, Stomopneustidae and Phymosomatidae were not studied. It is probable that the distribution and histochemistry of saleniid gut mucous cells would resemble those of family Arbacidae. Durham and Mellville (1957) have allied Mortensen's stirodont families Stomopneustidae and Phymosomatidae with the camarodonts; thus it would be interesting to see if the mucous cells in these two families have a stirodont or a camarodont type of distribution and histochemistry. In the order Camarodonta, only the family Parasaleniidae was not studied; it almost certainly should conform to the common camarodont pattern of mucous cell distribution and histochemistry.

Not all extant orders and families of irregular echinoids were covered in the present investigation. Not studied was the order Holectypoida; this order, which probably shares a common ancestor with the order Clypeasteroida, contains but one extant family, the Echinoneidae. The guts of echinoneids are much like those of clypeasteroids except that the lantern, along with the pharynx, is entirely missing in postlarval animals; presumably, therefore, only the esophagus should contain mucus cells. In the order Clypeasteroida, two families were not studied; presumably they would conform to the general clypeasteroid pattern of gut mucous cell distribution and histochemistry described in this paper. Also not studied was the order Cassiduloida; this order, which probably shares a common ancestor with the order Spatangoida, contains four extant families. Durham and Melville (1957) place cassiduloids closer to the amphisternate spatangoids than to the meridosternate spatangoids; it would, therefore, be interesting to see if cassiduloids have mucous cells limited to the distal esophagus instead of distributed throughout the proximal and distal esophagus. In the suborder Meridosternata of the order Spatangoida, two families were not studied and, in the suborder Amphisternata of the order Spatangoida, seven families were not studied. Presumably, the unstudied spatangoid families would conform to the meridosternate or amphisternate patterns of gut mucous cell distribution and histochemistry described in this paper.

We are indebted to Dr. Sandy Bruce, Dr. Rainer Martin, Dr. Andrew Packard, Dr. John S. Pearse, Mr. Robert Kiwala, Mr. George Lewbel and Miss Joane Molenock for the collection of some of the echinoids used in this investigation. Our thanks are further due to Dr. John S. Pearse for his critical reading of this paper and for his hospitality to one of us (N. D. H.) during a collecting trip to Egypt. We also wish to thank Mrs. Linda Holland and Miss Bessie Saghy for help with illustrations. It is a pleasure to acknowledge the cooperation of the administrations of the Stazione Zoologica di Napoli and of the U.W.I.-S.U.N.Y. Marine Laboratory at Discovery Bay, Jamaica during the course of this work.

SUMMARY

1. A standard terminology for echinoid gut regions is described such that one and the same name is applied to gut regions homologous among the regular echinoids, clypeasteroids and spatangoids.

2. In the regular echinoid order Aulodonta, all seven diadematid species studied lack a siphon, having instead a siphonal groove running the length of the stomach; previously, the only echinoid group known to lack a siphon was the regular echinoid order Cidaroidea.

3. All thirty-seven echinoid species investigated have gut mucous cells in the epithelium lining at least one region of the digestive tract; these cells contain sulfated acid mucus, nonsulfated acid mucus or neutral mucus.

4. Mucous cells of echinoid guts generally occur either individually as unicellular glands or collectively as secretory epithelial sheets; in addition, echinoid gut mucous cells occur (1) as intraepithelial glands in the esophageal epithelium of some species of the order Aulodonta and (2) as simple acinous glands invaginated into the connective tissue underlying the epithelium lining the distal esophagus in amphisternate species of the order Spatangoida.

5. Autoradiography of seven regular echinoid species reveals that, in mucous cells containing sulfated acid mucus, S^{35}-sulfate is incorporated in the basal-to-middle region of the cell, presumably into newly synthesized mucus; by 24 hr after administration of S^{35}-sulfate, the radioactive mucus migrates intracellularly toward the apex of the mucous cell.

6. The siphonal grooves of all cidaroids and aulodonts studied are free of mucous cells, as are the siphons of the other echinoid species studied.

7. In the regular echinoid order Cidaroidea, all four species studied have mucous cells in the pharynx, esophagus, stomach, intestine and rectum.

8. In the regular echinoid order Aulodonta, all seven species studied have mucous cells in the pharynx and esophagus; in five of these seven species, mucous cells are also found in the intestine and rectum.

9. In the regular echinoid orders Lepidocentroida, Stirodonta and Camarodonta (one, three, and fourteen species respectively studied) mucous cells are found in the pharynx and esophagus, but not in the stomach, intestine and rectum.

10. In the irregular echinoid order Clypeasteroida, all four species studied have mucous cells in the pharynx and esophagus, but not in the stomach, intestine and rectum.

11. In the irregular echinoid order Spatangoida, all four species studied have mucous cells restricted to the esophagus; these cells are in both the proximal and distal esophagus in the suborder Meridosternata, but are found only in the distal esophagus in the suborder Amphisternata.

12. The possible functions of echinoid gut mucus are discussed and the possible evolutionary history of selected features of the echinoid gut is traced from the Palaeozoic cidaroids to extant echinoids.

LITERATURE CITED

Anderson, J. M., 1966. Aspects of nutritional physiology, pp. 329–357. *In:* R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience Publishers, New York.

Bock, W. J., 1959. Preadaptation and multiple evolutionary pathways. *Evolution,* **13**: 194–211.

Buchanan, J. B., 1969. Feeding and the control of volume within the tests of regular sea urchins. *J. Zool.,* **159**: 51–64.

Casselman, W. G. B., 1959. *Histochemical Technique.* Methuen, London, 205 pp.

Chesher, R. H., 1963. The morphology and function of the frontal ambulacrum of *Moira atropos* (Echinoidea, Spatangoida). *Bull. Mar. Sci. Gulf Caribbean,* **13**: 549–573.

Chia, F. S., 1969. Some observations on the locomotion and feeding of the sand dollar, *Dendraster excentricus* (Eschscholtz). *J. Exp. Marine Biol. Ecol.,* **3**: 162–170.

Durham, J. W., 1966a. Phylogeny and Evolution, pp. U266–U269. *In:* R. C. Moore, Ed., *Treatise on Invertebrate Paleontology. Part U. Echinodermata 3* (*Vol. 1*), Univ. Kansas Press.

Durham, J. W., 1966b. Classification, pp. U270–U295. *In:* R. C. Moore, Ed., *Treatise on Invertebrate Paleontology, Part U. Echinodermata 3* (*Vol. 1*), Univ. Kansas Press.

Durham, J. W., and R. V. Melville, 1957. A classification of echinoids. *J. Paleontol.,* **31**: 242–272.

Fuji, A., 1961. Studies on the biology of the sea urchin. IV. Histological observation of the food canal of *Strongylocentrotus intermedius. Bull. Fac. Fish. Hokkaido Univ.,* **11**: 195–202.

Holland, N. D., and S. M. A. Nimitz, 1964. An autoradiographic and histochemical investigation of the gut mucopolysaccharides of the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.,* **127**: 280–293.

Humason, G. L., 1962. *Animal Tissue Techniques.* [*First edition*]. Freeman, San Francisco, 468 pp.

Koehler, R., 1883. Recherches sur les échinides des côtes de Provence. *Ann. Mus. Hist. Natur. Marseilles, Zool.,* 1 (No. 3): 1–139.

Leighton, D. L., 1968. A comparative study of food selection and nutrition in the abalone, *Haliotis rufescens* Swainson and the sea urchin *Strongylocentrotus purpuratus* (Stimpson). *Ph.D. dissertation, University of California at San Diego.*

Lewis, J. B., 1964. Feeding and digestion in the tropical sea urchin *Diadema antillarum* Philippi. *Can. J. Zool.,* **42**: 549–557.

Maximow, A. A., and W. Bloom, 1957. *A Textbook of Histology.* [*Seventh Edition.*] Saunders, Philadelphia, 628 pp.

McCormick, L., and R. C. Moore, 1966. Outline of classification. *In:* R. C. Moore, Ed., pp. U295–U297, *Treatise on Invertebrate Paleontology, Part U. Echinodermata 3* (*Vol. 1*), Univ. Kansas Press.

Mortensen, T., 1928. *A Monograph of the Echinoidea. I. Cidaroidea.* Reitzel, Copenhagen, 551 pp.

Mortensen, T., 1935. *A Monograph of the Echinoidea. II. Bothriocidaroida, Melonechinoida, Lepidocentroida, and Stirodonta.* Reitzel, Copenhagen, 647 pp.

Mortensen, T., 1940. *A Monograph of the Echinoidea. III. 1. Aulodonta. With additions to Vol. II* (*Lepidocentroida and Stirodonta*). Reitzel, Copenhagen, 370 pp.

Mortensen, T., 1943a. *A Monograph of the Echinoidea. III. 2. Camarodonta. I. Orthopsidae, Glyphocyphidae, Temnopleuridae and Toxopneustidae.* Reitzel, Copenhagen, 553 pp.

Mortensen, T., 1943b. *A Monograph of the Echinoidea. III. 3. Camarodonta. II. Echinidae, Strongylocentrotidae, Parasaleniidae, Echinometridae.* Reitzel, Copenhagen, 446 pp.

Mortensen, T., 1948. *A Monograph of the Echinoidea. IV. 2. Clypeasteroida. Arachnoididae, Fibulariidae, Laganidae and Scutellidae.* Reitzel, Copenhagen, 471.

Mortensen, T., 1950. *A Monograph of the Echinoidea. V. 1. Spatangoida. I. Protosternata, Meridosternata, Amphisternata I. Palaeopneustidae, Palaeostomatidae, Aëropsidae, Toxasteridae, Micrasteridae, Hemiasteridae.* Reitzel, Copenhagen, 432 pp.

Mortensen, T., 1951. *A Monograph of the Echinoidea. V. 2. Spatangoida. II. Amphisternata. II. Spatangidae, Loveniidae, Pericosmidae, Schizasteridae, Brissidae.* Reitzel, Copenhagen, 593 pp.

Nichols, D., 1959. The histology of the tube-feet and clavulae of *Echinocardium cordatum. Quart. J. Microscop. Sci.,* **100**: 73–87.

Pearse, A. G. E., 1960. *Histochemistry, Theoretical and Applied.* [*Second Edition.*] Churchill, London, 998 pp.

Prouho, H., 1887. Recherches sur le *Dorocidaris papillata* et quelques autres échinides de la Méditerranée. *Arch. Zool. Exp. Gén.* (Sér. 2), **5**: 213–380.

Reisman, A. W., 1965. The histology and anatomy of the intestinal tract of *Dendraster excentricus,* a clypeasteroid echinoid. *M.A. thesis, University of California at Los Angeles.*

Spicer, S. S., 1963. Histochemical differentiation of mammalian mucopolysaccharides. *Ann. New York Acad. Sci.,* **106**: 379–388.

Stott, F. C., 1955. The food canal of the sea-urchin *Echinus esculentus* L. and its functions. *Proc. Zool. Soc. London,* **125**: 63–86.

Reference: *Biol. Bull.*, **138**: 306–315. (June, 1970).

THE MATURATION OF *HYDROIDES DIANTHUS*[1]

DONALD E. LEONE

Environmental Sciences and Engineering Section, Research and Development Department, General Dynamics, Electric Boat Division, Groton, Connecticut, 06340

The most predominate technique used in marine fouling research is the evaluation of biological growths on immersion panels or test rafts exposed to the ocean environment (DePalma, 1964). A new approach to this problem is the development of a laboratory bio-assay procedure. A laboratory procedure in fouling research offers a technique which can provide reliable quantitative results rapidly under controlled conditions. An assay type test for marine fouling studies was first suggested by deSilva (1958) in his studies of the settling characteristics of the polychaete, *Spirorbis.* With *Spirorbis* as the assay organism, Meadows and Williams (1963) obtained results on substrate selectivity in twelve hour tests. Wisely (1963a, 1963b) and Freiberger and Cologer (1965) have also reported on the use of bio-assay techniques in marine fouling studies. In this regard the marine polychaete tubeworm, *Hydroides* (*Eupomatus*) *dianthus*, (Verrill, 1873), a common fouling organism, is a potential assay organism. A bio-assay procedure implies the mass rearing of the animal in the laboratory, the maintenance of mature adults, and the conditioning and maturation of adults for out of season spawning. Although some marine invertebrates have been well studied in the laboratory, (Loosanoff and Davis, 1950; 1951; 1963; Loosanoff, 1954; Moyse, 1960); little has been published concerning the mass rearing and maturation of the worm *H. dianthus* in the laboratory (Turner and Hanks, 1960; Wisely, 1958). The latter paper is concerned with *H. norvegica,* a closely related species.

The objective of this project was to examine the general problems pertinent to the maintenance of laboratory populations of *H. dianthus*, to maintain and culture suitable marine micro-algae as food types for worms, and to conduct multi-factorial experiments in determining the effects of temperature, food type, food concentration, salinity, and artificial sea water, on the maturation of the worm in the laboratory.

MATERIALS AND METHODS

Algal food

Four marine algal species were examined as food for the tubeworm; *Phaeodactylum tricornutum* (Bohlin), *Isochrysis galbana* (Parke), *Dicrateria inornata,* and *Nannochloris* sp. Cultures were obtained from Dr. R. Guillard, Woods Hole

---

[1] This paper is part of a thesis submitted in partial fulfillment of the requirements for the M.S. degree from the Graduate School, The University of Connecticut, Marine Research Laboratory, Noank, Connecticut. This project was supported in part by funds from the U. S. Navy Applied Sciences Laboratory, Brooklyn, New York, through a contract with Electric Boat Division, General Dynamics Corporation, Groton, Connecticut 06340.

Oceanographic Institution, Woods Hole, Massachusetts, and maintained in a growth medium recommended by Dr. Guillard, but modified slightly to include the following: (1) commercially prepared artificial sea water, (Rila Marine Mix, Teaneck, New Jersey; Instant Ocean, Aquarium Systems, Inc., Wickliffe, Ohio), (adjusted to 30‰ salinity) in lieu of natural sea water; (2) tris (hydroxy-methyl) amino methane buffer (1 g/l), and (3) a higher concentration of nitrate (204 mg/l as N).

Techniques used for the mass culture of algae were similar to those reported by Loosanoff and Davis (1963) and Ukeles (1965).

Maturation experiments

The maturation of worms in the artificial environment provided in the laboratory necessitates the study of the effects of some important environmental parameters on the worm. Some of these factors include temperature, food type, food concentration, salinity, and water type. Two multi-factorial experiments were planned in order to examine all the variables.

The first experiment (Experiment I) consisted of three separate factorial experiments. These involved three food types: *Phaeodactylum tricornutum*, *Isochrysis galbana*, and *Nannochloris* sp. Each was evaluated at three levels of supply. Two runs were made with each food type; one with artificial sea water (Rila Marine Mix), the other with natural sea water. Artificial sea water was prepared according to instructions supplied by the manufacturer. Natural sea water was obtained at Noank, Connecticut and was passed through a 0.45 micron membrane filter to remove natural food. Salinity was maintained at 30‰. At times it was necessary to increase the natural salinity of the sea water by heating and evaporation in order to achieve 30‰ salinity.

Temperature was maintained at three levels: 10° C, 16° C, and 22° C by temperature controlled aquaria. Each test beaker was aerated. The experimental variables tested in Experiment I are shown in Table I. Note that fifty-four tests were conducted and included two variables at three levels, two types of sea water, and three food types. The separate beakers were assigned levels of temperature, food concentration, and food type randomly.

Adult worms were maintained in a low-temperature aquaria (5° C) in natural sea water (30‰) for at least two weeks. The intent was to arrest or prevent gametogenesis. The sea water was continuously circulated through an ultraviolet irradiation chamber to keep bacterial populations to a minimum. No food was supplied to the worms during this period.

Prior to initiating an experimental run, four worms were taken from the preconditioning tank and removed from their tubes and examined for ova or sperm. In all cases the gametes were found to be immature or undeveloped.

A number of worms of approximately equal size were placed into two-liter beakers containing one liter of the appropriate medium (natural or artificial sea water) and the prescribed concentration of food added. Worms to be used at the higher temperatures were conditioned gradually to increasing temperatures at two day intervals until the desired temperatures were reached. At this point the factorial experiment was initiated. The sea water was changed and food pro-

TABLE I

Experimental variables evaluated—Experiment I

		Sea water type					
		Artificial			Natural		
		Food concentration (mg [dry] algae/worm/2 days)					
		C_1 (0.003)	C_2 (0.03)	C_3 (0.3)	C_1 (0.003)	C_2 (0.03)	C_3 (0.3)
Temperature (°C)	T_1 (10)	T_1C_1	T_1C_2	T_1C_3	T_1C_1	T_1C_2	T_1C_3
	T_2 (16)	T_2C_1	T_2C_2	T_2C_3	T_2C_1	T_2C_2	T_2C_3
	T_3 (22)	T_3C_1	T_3C_2	T_3C_3	T_3C_1	T_3C_2	T_3C_3

(a) In the experiment, three different food types were used: Run No. 1—*Phaeodactylum tricornutum*, Run No. 2—*Nannochloris* sp., Run No. 3—*Isochrysis galbana*. (b) Food concentration in the case of Run No. 1 was 0.02, 0.2, and 2.2 (mg [dry] algae/worm/2 days).

vided on alternate days for the duration of the experiment. Several worms were removed from each beaker and examined to determine the state of gametogenesis at intervals of approximately 12, 30, and 25 days.

Gonadial products were obtained by either removing the worm from its tube or breaking the tube and exposing the abdomen. Sexual products are immediately discharged from abdominal pores. Male products were a whitish discharge and females a pink discharge, (Grave and Oliphant, 1930; Wisely, 1958).

The following descriptive terms were used to describe the results:

NR—No Response—indicates absence of gametes
IO—Immature Ova—indicates small undeveloped ova (diameter less than 50 microns)
IS—Immature Sperm—indicates non-motile sperm
MO—Mature Ova—indicates mature ova (diameter greater than 50 microns)
MS—Mature Sperm—indicates motile sperm
PS—Probably Spent—indicates no response animals which were mature in a previous interval

A second multi-factorial experiment (Experiment II) was planned in order to evaluate the effect of salinity, and increased temperature levels on the maturation of the worm. This experiment involved three levels of temperature (22° C, 26° C, and 30° C), and three levels of salinity (15, 25, and 30‰). Three temperature controlled aquaria were utilized for the experiment. The sea water was artificial sea water (Instant Ocean) prepared at the three levels. Two variables at three levels or a total of nine combinations were tested. Two factorial experiments were managed simultaneously. *P. tricornutum* was used as the food organism in one factorial experiment; an algal mixture (*P. tricornutum, I.*

galbana, and *D. inornata*) was used in the other. Food was supplied at a concentration of 0.5 mg (dry) algae/worm/2 days.

Prior to initiating Experiment II, ten worms were examined for the presence of mature ova or motile sperm. In all cases the gametes were either immature or undeveloped.

The procedure for start-up of Experiment II was similar to that already described for Experiment I, except that worms were removed from beakers and examined for gametes at intervals of approximately 19, 24, and 31 days.

In addition to the descriptive response of maturity noted in Experiment I, male products were examined for motility, and counted in a Neubauer counting chamber. A drop of 10% formalinized artificial sea water was added to the sperm preparation in order to facilitate counting. Two counts were made and averaged. Female products were counted in a Sedwick-Rafter plankton chamber, and were sized and measured by a calibrated ocular micrometer. All sexual products were collected in either two or three ml of artificial sea water.

RESULTS

Experiment I

The results of the factorial experiments on conditioning of the worms fed *P. tricornutum, Nannochloris* sp. and *I. galbana* were summarized, grouped, and is presented in Table II. The per cent mature worms observed for each category was computed.

Gamete maturation was observed by the 12th day, but generally required 17 days or more. Some 30 per cent of the worms were mature by the 20th day and 37 per cent were mature by the 25th day.

TABLE II

Summary—Maturation experiment—Experiment I. effect of sea water, time, food, type, and temperature on maturation of Hydroides dianthus

	Mature	Immature	N. R.	P. S.	Number Total mature / number	Mature worms (%)
Sea water						
Artificial Sea water	54	92	16	19	54/181	29.8
Natural Sea water	37	77	30	21	37/165	22.4
Time (Days)						
12	17	78	25	—	17/120	14.2
20	39	61	14	17	39/131	29.8
25	35	30	7	23	35/95	36.9
Food type						
Phaeodactylum	23	45	6	3	23/77	29.9
Nannochloris	24	81	17	15	24/137	17.5
Isochrysis	44	43	23	22	44/132	33.2
Temperature (°C)						
10	8	76	17	9	8/110	7.3
16	22	65	17	6	22/110	20.0
22	61	28	12	25	61/126	48.4

At the highest temperature level tested in Experiment I (22° C), 48 per cent of the worms examined had mature gametes. At 16° C and 10° C, the percentage of worms that had matured was 20 per cent and 7 per cent, respectively.

Of the three algal food types examined, *Nannochloris* sp. was apparently the least effective. Both *I. galbana* and *P. tricornutum* were more effective in the maturation of the worm.

There was slightly better results in the gametogenesis of worms held in artificial sea water (30%) than in natural sea water (22%).

A summary of the data according to sex is shown in Table III. Of a total of 346 worms used in the experiment, 91 worms were mature (26%), and 255 worms were either immature or did not respond. From a total of 70 male worms observed, 29 were mature (41%), and from a total of 190 female worms observed 62 were mature (33%). Note that only 86 worms out of 346 did not respond or 75% of the worms responded in one way or another.

TABLE III

Summary—Maturation Experiment I. Response of the worms according to sex

	Males	Females	Total
Mature	29	62	91
Immature	41	128	169
No response	—	—	86
Grand total	70	190	346
% Mature	41	33	26

Experiment II

The results observed in the factorial Experiment II were summarized and the percent mature worms for each category was computed and is presented in Table IV.

At intervals of 19, 24, and 31 days, mature gametes were observed in 46, 62, and 64 per cent of the worms, respectively.

Low salinities affected worm maturation. Only 14% of the worms matured at 15‰ salinity, compared with 79% and 78% maturing at 25 and 35‰, respectively. Note that all deaths during the experiment occurred in those beakers having salinities of 15‰. In those worms surviving at 15‰ salinity, little or no tube growth was observed.

Maturation was not increased by subjecting worms to increasing temperature. Slightly more worms matured at the lower temperature (22° C) than did at the higher temperatures of 26° C and 30° C. Nevertheless, greater than 50% of the worms matured at all temperatures.

No difference in maturation was noted in feeding worms either *P. tricornutum* or an algal mixture. Worms matured fairly well fed either type of food. Table IV also presents maturation data based on the response of female and male worms. Generally, no significant changes occurred, except that increasing temperatures and a mixed food enhanced the maturation of females, but not males.

TABLE IV

Summary—Maturation experiment—Experiment II. Effect of food type, salinity, temperature, and time on maturation of Hydroides dianthus

	Mat.	Im.	N. R.	Dead	No. mat. / Total number	Mature worm (%)	Mature males (%)	Mature females (%)
Food type								
Mix	42	9	14	11	42/76	55.2	45.3	78.4
Phaeo	50	16	16	12	50/89	56.1	53.8	59.4
Salinity (‰)								
15	8	16	11	23	8/58	13.8	14.6	11.7
25	44	3	9	—	44/56	78.5	72.2	90.0
35	40	6	5	—	40/51	78.4	71.4	86.9
Temperature (°C)								
22	33	15	9	1	33/58	57.0	63.3	50.0
26	27	7	13	1	27/48	56.2	41.4	78.9
30	32	3	3	21	32/59	54.3	45.7	84.5
Time (Days ± 1)								
19	31	7	7	23	31/68	45.5	36.0	72.2
24	28	10	7	—	28/45	62.2	64.0	60.0
31	33	8	11	—	33/52	63.5	60.0	68.1

Code: Mat. = Mature worms; Im. = Immature worms; N.R. = No response.

A summary of the data according to sex is shown in Table V. Of a total of 138 worms used in this experiment, excluding dead animals, 92 (67%) were mature. Mature gametes were obtained from 52 male worms (93%) and 40 female worms (66%). Of 138 worms observed only 21 worms did not respond or 85% of the worms did respond in some manner.

TABLE V

Summary—Maturation Experiment II. Response of worms according to sex

	Males	Females	Total
Mature	52	40	92
Immature	4	21	25
No response	—	—	21
Dead	—	—	23
Grand total	56	61	161
% Mature	93	66	67*

* Excludes dead animals.

Fecundity of Hydroides dianthus *(Experiment II)*. The gametes released were collected and counted, either in a Neubauer counting chamber, or Sedgewick-Rafter plankton counting chamber. The values obtained in the case of males represent an average of two counts, however, in the case of females, all the ova in a one ml sample were counted. The data concerning gamete productivity were grouped, averaged, and summarized (Table VI).

Most sperm were obtained in those worms examined by the 19th day. Worms observed by the 24th and 31st day, resulted in decreasing numbers of motile

TABLE VI

Summary—Effect of time, temperature, salinity, and food type, on number of mature gametes obtained in laboratory spawning of Hydroides dianthus

	Sex	No. worms	Average no. mature gametes/worm
Time (Days ± 1)			
19	M	17	93,361,176
	F	8	12,208
24	M	16	65,870,187
	F	12	18,850
31	M	18	36,688,888
	F	15	49,975
Temperature (°C)			
22	M	19	92,150,000
	F	9	41,371
26	M	12	41,727,915
	F	15	37,006
30	M	20	52,493,750
	F	11	13,278
Salinity (‰)			
15	M	6	6,341,666
	F	2	13,162
25	M	25	78,462,920
	F	16	28,329
35	M	20	65,232,000
	F	17	34,935
Food type			
Mix	M	24	73,777,833
	F	16	27,580
Phaeo	M	27	49,288,703
	F	19	33,274

sperm present. The number of ova observed, however, increased with each increasing time interval.

Nearly twice as many sperm were obtained from worms matured at 22° C than at either 26° C or 30° C. The number of ova obtained in worms matured at 22° C and 26° C and were not significantly different, approximately 40,000 ova/worm; however, at 30° C only approximately 13,000 ova/worm were obtained.

The lowest salinity tested obviously affected gamete production. Small numbers of ova and sperm were attained at the 15‰ salinity. At the 25‰ salinity, some 78 million sperm/worm were obtained compared with 65 million sperm/worm at 35‰ salinity. With the mature ova, more ova were obtained at increasing salinities.

Food type has an effect on the number of sperm released. More sperm were obtained in those worms fed an algal mixture, than from those worms fed a single species. The ova did not appear to be grossly affected by the different food types, although slightly higher values were recorded in those worms fed only the diatom.

The male releases about two orders of magnitude greater numbers of sperm than do females releasing ova. The average male released some 62 million sperm while the average female released some 30 thousand mature ova.

Discussion

The two multi-factorial experiments represented an effort to determine the influences of algal food type, temperature, salinity, water type, and conditioning time on the maturation of *H. dianthus* in the laboratory.

Artificial sea water prepared from commercially available synthetic sea water mixtures had little effect on maturation. Effective maturation occurred in the artificial sea water as well as in the natural sea water. The use of artificial sea water in the laboratory culture and maturation of the worm would be advantageous. It eliminates the problem of variability and uncertainty of natural sea water composition.

Mature gametes were observed in some instances in twelve days, however, a greater number of worms indicated maturity in twenty-five days. Increasing the conditioning time to thirty-one days did not yield more mature worms. In studies reported by Turner and Hanks (1960), maturation of *H. dianthus* was achieved between seven to ten days at 23° C in natural sea water.

All food types examined as food were effective in the maturation of the worm. *Nannochloris* sp. the smallest alga under study, was apparently the least effective. An algal mixture did appear to effect the maturation of female worms (Table IV).

Temperature affects gametogenesis. The per cent of mature worms was highest in those worms held at 22° C, and decreasing values were obtained with decreasing temperatures. Increasing temperature above 22° C did not enhance maturation of the male worms, highest values were attained with the 22° C level. However, female worm maturation was increased by increasing temperature (Table IV).

Low salinities affected worm survival and worm development. All deaths occurring in the experiment were observed at 15‰ salinity. The high temperature (30° C) and low salinity (15‰) accounted for 21 of the 23 deaths recorded. In a study with mollusc larvae, Davis and Calabrese (1964) reported that salinity affects temperature tolerance of these forms. Neff (1968), reports that *H. dianthus* is unable to produce mineral for tube building, when salinities are below 20‰. The low salinity level selected for evaluation was obviously unsatisfactory for the maturation and maintenance of the worm in the laboratory. The maturation of the worm was satisfactory in the range of 25–35‰.

An analysis of variance on the effect of temperature and salinity on male and female worms matured in Experiment II was completed. The effects of type of food and conditioning time were ignored for purposes of the analysis. The per cent mature worms were transformed by an arcsine transform to eliminate zero percentages. Female gamete maturation was significantly affected by both temperature and salinity, at the 5% probability level. The interaction was non-significant. Temperature, salinity, and interaction effects were all non-significant in the case of male gamete maturation.

Of a total of 507 worms used in both experiments, 107 worms did not respond and only 23 deaths were recorded, or approximately 75% of the worms responded to the conditioning treatments. Remarkable response despite the use of wide ranges of temperature, food concentration, and salinities.

Mature gametes were observed in 41% of the males and 33% of the females in Experiment I, and in 93% of the males, and 66% of the females in Experiment II, only 16% of the worms were immature in Experiment II indicating that better conditions for maturation existed in Experiment II.

An estimate of the number of mature gametes released by *H. dianthus* is of interest. No information concerning gamete production was found in the literature. Tubeworm ova production was decreased by increasing temperature but increased by increasing salinity. Most mature ova were obtained in a conditioning time of 31 days, with decreasing values at 25 to 19 days, respectfully. The average number of mature ova released by a single worm was approximately 30 thousand. This represents a value averaged over all the conditions examined in the experiment. The number ranged from less than 100 to approximately 200 thousand ova/worm. Ova production in worms appears to be for less than that reported for molluscs. Davis and Chanley (1956), report that oysters produce some 50 million ova, and clams about 25 million ova.

The average number of motile sperm released by a single worm was 62 million. The number of sperm released ranged from 765,000 to greater than 400 million/worm.

In general, this study provides guide lines and ranges to permit the maintenance, culture, and maturation of the tubeworm *H. dianthus* in the laboratory. Optimum conditions were not achieved, yet, a high degree of maturation was accomplished in the laboratory environment.

The author is indebted to Drs. R. J. Benoit, E. A. Zuraw, T. Gaucher, and J. S. Rankin, for their advice, criticism, encouragement, and discussions.

SUMMARY

1. All microalgae used as food were satisfactory food for the worm. (a.) *I. galbana* and *P. tricornutum* were better food organisms than was *Nannochloris* sp. (b.) An algal mixture of *I. galbana, D. inornata* and *P. tricornutum* proved to be slightly better than the single food species *P. tricornutum.* (c.) No effect of feeding level was observed. An algal food concentration of 0.25 mg (dry weight) algae/worm/day appears satisfactory.

2. Artificial sea water did not inhibit maturation.

3. Gametogenesis was directly related to temperature up to 22° C, the higher the temperature the greater the number of sexually mature worms. The number of mature worms was not increased appreciably above 22° C.

4. Worms were mature in 12 days of conditioning, however, more worms indicated maturity in 25 days.

5. Low salinity (15‰) affected worm survival and worm maturation.

6. A female worm expels an average of 30 thousand mature ova. The male worm releases about 62 million sperm. (a.) Sperm and ova production was decreased by high temperature (30° C), and low salinity; ova production increased with time but sperm production decreased with time. (b.) Fecundity was greater in worms fed an algal mixture.

LITERATURE CITED

Davis, H. C., and P. E. Chanley, 1956. Spawning and egg production of oysters and clams. *Biol. Bull.*, **110**: 117–128.

Davis, H. C., and A. Calabrese, 1964. Combined effects of temperature and salinity on development of eggs and growth of larvae of *M. mercenaria* and *C. virginica*. *Fishery Bull. Fish Wildlife Serv. U. S.*, **63**: 643–655.

DePalma, J. R., 1964. Marine fouling research, a state-of-the-art report. *Information manuscript Report No. 0-1-64.* Marine Sciences Dept., U. S. Naval Oceanographic Office, Washington, D. C.

deSilva, P. H. D., 1958. Use of artificially librated larvae in experiments on the settling behavior of *Spirorbis*. *Nature*, **182**: 1751–1752.

Freiberger, A., and C. P. Cologer, 1965. Rearing barnacles in the laboratory, a new approach to the old problem of marine fouling. *Nav. Res. Rev.*, **18**: 8–13.

Grave, B. H., and J. F. Oliphant, 1930. The longevity of unfertilized gametes. *Biol. Bull.*, **59**: 233–239.

Loosanoff, V. L., and H. C. Davis, 1950. Conditioning *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. *Biol. Bull.*, **98**: 60–65.

Loosanoff, V. L., and H. C. Davis, 1951. Delaying spawning of Lamellibranchs by low temperatures. *J. Mar. Res.*, **10**: 197–202.

Loosanoff, V. L., 1954. New advances in the study of bivalve larvae. *Amer. Sci.*, **42**: 607–624.

Loosanoff, V. L., and H. C. Davis, 1963. Rearing of bivalve molluscs. *Advan. Mar. Biol.*, **1**: 1–136.

Meadows, P. S., and G. B. Williams, 1963. Settlement of *Spirorbis borealis* (Daudin) larvae on surfaces bearing films of microorganisms. *Nature*, **198**: 610–611.

Moyse, J., 1960. Mass rearing of barnacles in the laboratory. *Nature*, **185**: 120.

Neff, J. M., 1968. Calcium carbonate tube formation by serpulid polychaete worms: Physiology and Ultrastructure. *Ph.D. thesis, Duke University*, North Carolina, 306 pp.

Shearer, C., 1911. On the development and structure of the trochophore of *Hydroides uncinatus* (Eupomatus). *Quart. J. Microscop. Sci.*, **56**: 543–591.

Turner, H. J., and J. H. Hanks, 1960. Experimental stimulation of gametogenesis in *Hydroides dianthus* and *Pecten irradians* during the winter. *Biol. Bull.*, **119**: 145–152.

Ukeles, R., 1965. A simple method for the mass culture of marine algae. *Limnol. Oceanogr.*, **10**: 492–495.

Verrill, A. E., 1873. Report upon the invertebrate animals of Vineyard Sound and adjacent waters, with an account of the physical characters of the region. *U. S. Comm. Fish. Wash. Rep.*, **1871–1872**: 295–778.

Wisely, B., 1958. The development and settling of a serpulid worm *Hydroides norvegica* (Gunn) (Polychaeta). *Australian J. Mar. Freshwater Res.*, **9**: 351–361.

Wisely, B., 1963a. Detection and avoidance of a cuprous oxide antifouling paint by bivalve and gastropod larvae (Mollusca). *Australian J. Mar. Freshwater Res.*, **14**: 60–69.

Wisely, B., 1963b. Effects of antifouling paints on settling larvae of the bryozoan *Bugula neritina*. *Australian J. Mar. Freshwater Res.*, **14**: 44–59.

Reference: *Biol. Bull.*, **138**: 316-325. (June, 1970).

BURROWING BEHAVIOR OF THE SEA ANEMONE *PHYLLACTIS*

DOROTHEA CASKEY MANGUM[1]

Department of Biology, University of Arizona, Tucson, Arizona 85721

There are a number of sea anemones from different families of actinarians that are known to burrow into sand or mud flats. Burrowing is not an aberrant or unique behavior of anemones, but a frequently observed activity. The general characteristics of burrowing have been described by Stephenson (1928), and Ansell and Trueman (1968) have described the fluid dynamics of burrowing in the sea anemone, *Peachia hastata* Gosse. The following study was undertaken in order to explain burrowing behavior in terms of the neuromuscular organization of the anemone, *Phyllactis concinnata,* and to compare burrowing with other well known anemone activities (Batham and Pantin, 1950b, 1954; Ross and Sutton, 1967; Pantin, 1952).

MATERIALS AND METHODS

The anemone, *Phyllactis concinnata,* is a member of the family Actinidae found in the Gulf of California and Calloa, Peru (Carlgren, 1949, 1951). The anemones were collected from intertidal sand flats in Cholla Bay, a locality near Puerto Penasco, Sonora, Mexico. They ranged in size from ½ inch to 2½ inches across the oral disc, and from 1 inch to 6 inches in length when the column was expanded. They were maintained in the laboratory in twenty-gallon salt water aquaria with a coarse sand and shell hash substrate.

A Grass S. D. 5 stimulator with insulated platinum electrodes was used to give electric shocks. Stimulus duration was 2 milliseconds, voltage was twice threshold, and stimulus frequency was varied depending on the purpose of the experiment. Three methods were employed to analyze the behavior of these anemones: direct observation, photography of the anemone in various stages of burrowing and the recording of movements with a pen-writer. When recording, three channels of a four channel E and M Physiograph were used to monitor movement while the fourth served as a time base. The motion transducers were isotonic A-myographs to which long lever arms were attached and lightly counterbalanced in order to exert minimum tension on the anemone. One end of a monofilament thread was tied to the end of the lever and the other attached to a thread sewn to the column of the anemone. Extensive anemone muscle movements could be recorded without the digging performance of the anemone being appreciably affected by the slight change in the tension caused by the depression of the myograph spring. The majority of the recordings were made from anemones that had three thread loop attachments sewn to the sphincter muscles. The attachments were made at equal distances around the column as determined by counting the mesentary insertions. After an anemone had been attached to the levers, it was placed in a one-gallon aquarium filled with sea water with a two-inch layer of sand on the bottom. The water was aerated continuously and temperature was maintained between 20.5° and 22° C. In most experiments the physiograph records were accompanied by direct observations over long periods of time in order to attribute the appropriate activity to recorded movements. Certainly the effect of tying anemones to levers cannot be discounted, but observations in the laboratory and in the field indicate that such behavior as digging is little changed by attachments.

---

[1] Present address: Box 786, Angleton, Texas 77515.

Results

Gross morphology

Carlgren (1951) has given a description of *Phyllactis concinnata* found in the Gulf of California. Gross dissections and examinations of histological sections during this study confirm his findings and emphasize the following points. The column is smooth except for 40 to 48 longitudinal rows of verrucae at the top. These are small adhesive vesicles which adhere to bits of shell and sand grains. Above the verrucae lies a ruff or collar which may be as much as 4 inches in diameter. The ruff is characteristic of the genus and is formed of fronds which bear a variable number of papillae. It is very sensitive to tactile stimuli and may also serve a chemosensory function. The oral disc is small, and surrounded by 40 to 48 tentacles. The pedal disc is well developed, an unusual feature in a borrowing anemone. Internally, there are usually two siphonoglyphs and 20 to 24 pairs of perfect mesenteries. The strong longitudinal retractors are located on the endocoelic surface of all mesenteries except the directives. On the opposite side of each mesentery there is a thin sheet of transverse radial fibers. Growing over these in the bottom half of the column are well developed parieto-basilar muscles. There are strong parietals, which run longitudinally on each side of the mesenterial attachment of the column, and form a continuous band with the parieto-basilars in the upper part of the column. Circular muscles line the column and oral and pedal discs. There is a weak sphincter muscle below the ruff.

Compared to the musculature of non-burrowing forms, that found in the borrowing anemones differs in development rather than in kind of muscles. The burrowing anemones have very powerful retractor muscles to execute a quick withdrawal (Batham and Pantin, 1950a). The sphincter muscle of these anemones is not well developed, and the only method by which one can produce a sphincter closure is by excessive electrical stimulation. The sphincter muscle takes part in the fast closure reflex of both *Metridium* and *Calliactis* (Robson, 1961). The *Phyllactis* does not exhibit this protective closure of the oral disc. As in *Stomphia* (Sund, 1958), the parieto-basilar muscles of *Phyllactis* are well developed and serve an important function in the attachment and movement of the pedal disc.

Histology of the nervous system

Some success was attained in staining the nervous system of *Phallactis* with methylene blue following Pantin's (1952) method. There is a comparatively dense network of large bipolar cells on the surface of the retractor muscle. The length of these cells was determined by observing the freshly stained preparations with an ocular micrometer, and the maximum length seen was 6 mm in the fully relaxed muscle. In preparations where the muscles were not fully relaxed, the cells appeared to be as long as in a relaxed one, but their course on the surface above the folds of the muscle was not as direct and they were more difficult to measure. The nerve net is sparse on the radial surface of the mesentery. No multipolar cells are found on the mesentery, and the column has few bipolar cells and no multipolar cells. Sense cells are small but numerous, particularly at the junctions of the mesenteries and the column, in the oral disc, and in the tentacles. The nerve net of *Phyllactis* does not appear to be basically different from that of *Metridium* or *Calliactis.*

Responses to electrical stimuli

The Phyllactis exhibits the characteristic facilitated response that has been described for other anemones (Pantin, 1935; Pantin and Vianna Dias, 1952). There is normally no response to a single shock applied to any part of the column or ruff. However, with repetitive stimulation there is a symmetrical shortening of the column to each shock after the first if the interval between stimuli is shorter than 1.5 seconds at 21° C. As in other anemones, this response is considered to be brought about primarily by the contraction of the retractor muscles.

In *Phyllactis* the facilitating effect of the stimulus is influenced by the temperature. At 10° C the longest interval between the first stimulus and a second which will produce 5% of the maximum response is 3 seconds, at 21° C the interval is 1.5 seconds, and at 32° C it is less than a second. These values are within the range of those recorded for *Metridium* and *Bunodactis* (Pantin and Vianna Dias, 1952). As is true for *Bunodactis* there is no obvious adaptation of the facilitation rate of *Phyllactis* to the tropical temperature range.

Parietal contractions have been studied quite extensively in *Metridium* (Batham and Pantin, 1950b), *Calliactis* (Needler and Ross, 1958), and *Bunodactis* (Pantin and Vianna Dias, 1952). These contractions of the column are slow and have a variable latent period. *Phyllactis* also exhibits these typical slow responses when shocks are given at too low a frequency to elicit a retractor response. Another response that occurs frequently is elicited by several stimuli at a low frequency or by a single shock applied to the side of the column. Then there is a sharp bending of the column usually towards the stimulating electrodes and it would appear that bending is due to a contraction of the parieto-basilar muscles. A similar response to a single stimulus is seen in *Stomphia* and is thought to be a contraction of the parieto-basilar muscle (Hoyle, 1960).

Analysis of the burrowing behavior

The buried anemone. Phyllactis buried in the sand flats of Cholla Bay has only the ruff, oral disc and tentacles exposed. When the tide is out the ruff and oral disc may be covered with sand, or withdrawn into the burrow. When the tide starts to come in, the anemones elongate until the oral disc is level with the top of the burrow again. Strong mechanical stimuli will cause the anemone to withdraw. In the laboratory greatest activity can be evoked when live brine shrimp are added to aquaria, at which time the tentacles are extended and wave about.

Apart from these feeding movements and periodic elongations and withdrawals, anemones are not very active in a burrow. Physiograph records confirm this and show that buried anemones exhibit arhythmic activity.

The burrowing anemone. A *Phyllactis* placed on any of a variety of sand substrates starts digging in a few minutes. Burrowing is initiated when the anemone is in a contracted state and upright on the substrate. Muscle contractions associated with digging are rhythmical, and a number of muscle systems are intimately involved. In brief, digging appears to be accomplished by a shortening of the column which acts as a hydrostatic skeleton and forces fluid into the inflated pedal disc and the disc into the sand. This is followed by an elongation of the column as the animal prepares for the next contraction. The anemone continues to dig until it reaches hard substratum, either a rock or large shell fragments. The amount of time required for an anemone to dig in completely varies with the anemone, the temperature and the substrate, but under ordinary environmental conditions it takes less than 2 hours. The rhythmic contractions cease once the anemone is attached to the substratum.

The various phases of burrowing behavior are diagramed in Figure 1. A shortening of the column (Fig. 1A) may be regarded as the first step in burrowing. Typically such a movement is said to be due to parietal activity (Pantin, 1952; Batham and Pantin, 1954). However, if the pedal disc is hooked up to one myograph and the mid-column and the sphincter to the other two, the physiograph record shows that the first contraction in the pedal disc occurs approximately 30

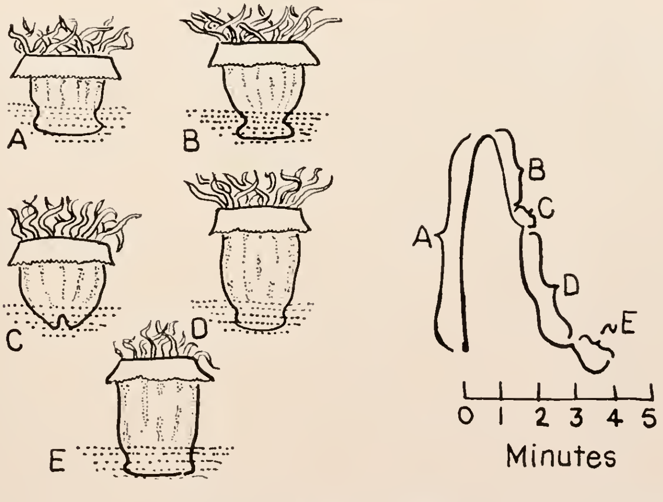


FIGURE 1. On the left is the diagrammatic representation of the various phases of burrowing behavior in the sea anemone, *Phyllactis concinnata.* "A" is the shortening of the column, which is followed by a circular muscle contraction passing down the column as shown in "B," and in "C" the pedal disc constricts. Then the pedal disc begins to enlarge and the column lengthens in "D" and "E." On the right is a physiograph tracing representing a single contraction as recorded from one of three attachments to the column. The brackets "A" through "E" show how the tracing is correlated with the burrowing phases shown on the left. As the column contracts, there is an upward movement of the pen as seen in "A," and the column elongates and the pen moves downward as seen in "B" through "E."

seconds before any in the column or ruff. This would seem to indicate that the parietal-basilar muscles contract in unison first and this action is followed by a simultaneous contraction of all parietals. There must also be a slow contraction of the retractors for the oral disc to pull down symmetrically (Batham and Pantin, 1954).

Following column shortening, there is elongation as the parietals and parietal-basilars relax and a peristaltic wave of circular muscle contraction moves toward the pedal disc (Fig. 1B). The circular muscle contraction appears as a band or as a wave of increased tone moving down the column and, as has been noted (Batham and Pantin, 1951), it effectively causes the elongation of the column. When the circular muscle contraction reaches the pedal disc, which is quite inflated during the parietal contractions, the latter constricts and assumes a concave shape (Fig. 1C). However, within 30 seconds to a minute the pedal disc begins to enlarge again and to assume a convex shape as water flows in and the parieto-basilar and circular muscles relax (Fig. 1D). This latter movement detaches the pedal disc from any rock or shell to which it may have adhered during the contraction of the parietals. Once the pedal disc is fully inflated, the parieto-basilar contractions reoccur, followed by the parietal contractions. Some records show an occasional quick contraction of the retractor muscle during digging. This is a random event which occurs after the animal has started digging. Such a contraction forces water out of the column through the anemone's mouth and makes the anemone smaller. The smaller anemone then settles into a larger hole. A quick contraction often occurs upon the cessation of the rhythmic digging movements as well.

It appears that the integrity of the hydrostatic skeleton must be maintained. If the column is damaged or cut in any way, the anemone will not dig in, and will eventually die. Cuts in the pedal disc do not prevent burrowing, presumably because holes can be sealed by muscular contraction, whereas weak muscles of the column are ineffective in preventing fluid loss.

Physiograph records of a digging anemone show the time course and extent of movement during each phase of the digging period. Phase "A," (Fig. 1) represents the shortening of the whole column as the parieto-basilar muscles and the parietals contract simultaneously. The subsequent change in slope marked "B," represents the relaxation of the parietals and the elongation of the column due to the peristaltic wave. There is a plateau at "C," sometimes with a slight peak. This represents the time when the anemone settles into the substrate as the peristaltic wave reaches the pedal disc and the latter deflates. "D" represents further elongation of the column as there is an increase in tone in the circular muscle and the pedal disc fills with water. This is the stage preceding the next parietal contraction. In some recordings there is a fifth phase, "E" which represents a final elongation occurring immediately before contraction.

The rhythmic quality of the contractions is shown in Figure 2. There is a simultaneous contraction of all parietal muscles during digging. Physiograph records of the digging movement of different anemones are quite similar, not only in pattern, but in time course. In more than 30 records from anemones collected at different times of the year, the most frequently measured interval between the parietal contractions was 4 minutes at 21°C. The interval between contractions

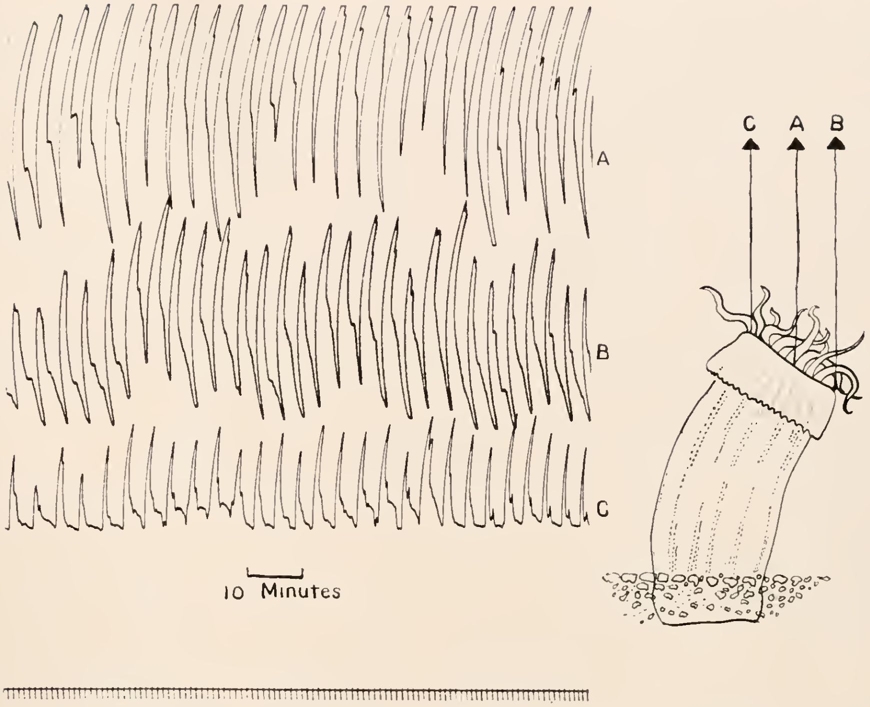


FIGURE 2. A portion of an actual physiograph record from a burrowing *Phyllactis* with three attachments on the column. Each contraction of the column caused all three pens to move upward simultaneously. In this instance the anemone was at a slight angle, and tracings "A" and "B" show more amplitude because of this. This portion of the record is nearly 2 hours long and the consistency of the rhythmic contractions is clearly seen.

may be somewhat longer or shorter at the beginning or the end of the digging activity, however.

Burrowing in *Peachia hastata* Gosse (Ansell and Trueman, 1968) is a similar process, but this anemone has a physa instead of a pedal disc. When the anemone is burrowing the physa undergoes a cyclical change of shape as "introversion and then eversion" occurs. This change of shape is due to the peristaltic contraction of the circular muscles, which is followed by contraction of the longitudinal musculature and which results in fluid being forced into the physa. According to Ansell and Trueman, the physa serves as a "physal anchor" during the column contraction. Also, the physa starts the initial penetration at the onset of burrowing, as the anemone lies on the substrate. After about $\frac{1}{3}$ of the anemone has penetrated the sand, the cyclical change of shape of the physa is accompanied by sharp contractions of the column musculature. These contractions do not occur with each introversion and eversion cycle, and the interval between the contractions increases as the burrowing progresses. This is in contrast with the *Phyllactis* where a major contraction of the column occurs with each inflation of the pedal disc.

Specimens of *Phyllactis* were observed after they were released in their natural habitat. Anemones placed in a small tidepool began to dig within 10 minutes. By the time an hour had passed they were quite well dug into the substrate. As far as could be determined the burrowing movements of these animals in the natural habitat were the same as those of the anemones in the laboratory. On the other hand, specimens of *Phyllactis* released in open waters were rolled about on the sand and were unable to burrow.

The effect of food on the burrowing behavior. Five of fifteen specimens of *Phyllactis* buried in an aquarium were taken out of the sand and then placed back on the surface. None of the fifteen had been fed for a week. Once the five started digging actively some brine shimp were released into the water. Only the buried anemones showed feeding activities, and they would catch as many as 10 shrimp each before they stopped feeding. On the other hand, brine shrimp that swam into the tentacles of digging anemones would not be caught and held. These experiments suggest that there is a change in threshold for nematocyst discharge during digging, or an inhibition of the entire feeding process. A similar response is reported by Ross and Sutton (1964) for *Stomphia.*

The effects of mechanical and electrical stimuli on a digging anemone. Anemones pulled out of the sand after they start burrowing will resume digging if placed on the sand, or will continue contracting rhythmically if placed on a hard substratum such as a glass plate. In the latter instance the rhythmic contractions become part of a pedal locomotion, allowing the anemone to move off of the plate and dig back into the sand. Mechanical stimuli of different strengths applied with a glass rod elicit responses ranging from the slight twitch of a tentacle to complete withdrawal (retractor response) of an anemone that is in a burrow. Light touches do not effect the overall behavior of a digging anemone. On the other hand, a strong stimulus will evoke a retractor response during any phase of the digging. However, digging anemones recover from this stimulus quickly and continue their rhythmic activity.

On no occasion did electrical stimuli of any sort elicit rhythmic digging behavior in an inactive anemone lying on the surface of the sand. Electrical stimuli did have some effect on burrowing animals, however. A series of stimuli were given to digging animals during various phases of the digging activity at a frequency and voltage that would elicit either a parietal or a retractor response in a non-burrowing animal. The results show that often a parietal response will occur during the contraction or relaxation phase of burrowing if shocks are given at a low frequency, and the rhythmic pattern of digging is not upset. A retractor response is produced by shocks of sufficient frequency during any phase of the burrowing, and if the retractor response occurs during the column contraction it does not alter digging behavior. However, a retractor response during the "C" or relaxation phase seems to increase the extent of the relaxation before the next contraction.

DISCUSSION

The burrowing behavior is another example of the way in which the sea anemones carry out a coordinated activity. The rhythmic contractions of the longitudinal and circular muscles of the column manipulate the fluids of the column's hydrostatic skeleton so that the pedal disc is pushed into the substrate. One of the most interesting features of this behavior is the very rhythmic sustained contractions of the column. In studies of more than 30 anemones of different sizes, the 4-minute interval between contractions was remarkably consistent, and rhythmic contractions could be maintained for as long as 6 hours. Burrowing behavior is probably under the regulation of a system which controls the timing and sequence of each muscle contraction. There is evidence that the control system not only initiates burrowing and acts positively to keep it going, but that it inhibits other activities, such as feeding behavior, as well. There are two possibilities for such a control system. One would be that each contraction of the column could be dependent on the excitation set up by the preceding, as in a chain reflex; the other would be that the control system involved a pacemaker.

It appears that the control system does involve a pacemaker. This is the best explanation for the consistent interval between contractions in the different anemones. If the contractions were under the control of a chain reflex, it would seem that the interval would vary from one anemone to another. Since the spread of the excitation is slow, there would be a longer interval between contractions in an anemone with a column measuring 6 inches than in one with a column that measures 1 inch, but this is not true. The continuation of the rhythmic contractions when the anemone is pulled out of one substrate (sand) and put on another (glass) is best explained by a pacemaker system. Furthermore, the contraction interval is not affected by a variety of substrates. If the burrowing were controlled by a chain of reflexes, and if one substrate could be penetrated more readily than another, each step in the behavior could be completed sooner and the interval between the contractions would be shortened; this is not the case. The rhythmicity of burrowing behavior is not unique to *Phyllactis.* Ansell and Trueman (1968) report that a burrowing *Peachia hastata* Gosse has rhythmic contractions of the column musculature with intervals of usually 1½ minutes. It is difficult to pinpoint the location of the assumed pacemaker or pacemakers in the *Phyllactis.* Multipolar cells that are suggested as a pacemaker system for *Stomphia* (Robson, 1963) are not obvious in the column.

Pantin (1952) described a number of different functions for the column "action system" of anemones, including maintenance of shape, movement in feeding and locomotion. Burrowing is another adaptation of the column action system. A control system with its pacemaker element in a burrowing anemone could be similar to one that permits other anemones to undertake pedal locomotion. My own studies (unpublished) of another species of *Phyllactis* and *Bunodosoma cavernata* show that column contractions of these anemones are rhythmic as they move laterally along the surface and this is remarkably similar to pedal locomotion in *Metridium* (Batham and Pantin, 1950c), and *Stomphia* (Ross and Sutton, 1967). Pantin (1965) suggested that rhythms as in the normal parietal-circular muscle contractions of *Metridium* may have origins from local pacemakers. Slight modifications of this basic capacity for rhythmic coordinated activity has permitted anemones to develop a varied behavior. Further study of the nature and origin of this basic rhythmicity could be made by taking advantage of the predictable, consistent and sustained nature of the rhythmicity found in the burrowing behavior of anemones.

It is not known if these anemones, when in their natural environment, move out of the sand and then burrow again. *Phyllactis* in the laboratory aquaria will stay in one location for years, as long as they are undisturbed and the water is well filtered and aerated. In their natural environment *Phyllactis* might be washed out of their burrow by a severe storm. The fact that burrowing activities can be initiated quickly would be of adaptive advantage when this occurs. Digging is not triggered by a specific stimulus as is the swimming behavior of *Stomphia* (Robson, 1961), but is activated by a set of conditions much as the locomotor phase of *Metridium* (Batham and Pantin, 1950c). There are fewer conditions for digging than for locomotion however, in fact, only three. The pedal disc must be unattached, there must not be any sand around the column, and there must be a soft substrate available. Cessation of burrowing occurs when the column is completely buried and the pedal disc is attached to a firm substrate. Other marine invertebrates burrow by pushing a rhythmically contracting foot or proboscis into the sand (Trueman, 1967). It is interesting that anemones accomplish the same thing albeit at a slower rate, but with a much less complex organization of neuromuscular tissue.

The author is grateful to Dr. Peter E. Pickens for his suggestions and encouragement during the course of this study and to him and to Dr. Elaine A. Robson for helpful criticism of this manuscript. The author is also very much indebted to Mr. and Mrs. Walter M. Vreeland, Jr. for the use of their facilities at Cholla Bay, Sonora, Mexico and for their assistance in many ways and to Mrs. Joy W. Cooper for undertaking the histological work. The anemone was identified with the kind assistance of Mr. Charles Cuttress. This work was supported by a predoctoral training grant (USPHS #2 T 1-GM 441-04) and a NSF grant to Dr. Peter E. Pickens (NSF #GB-3745).

SUMMARY

1. The burrowing behavior of the anemone *Phyllactis* is a very consistent and predictable activity.

2. Burrowing is accomplished by rhythmic contractions of the column musculature around a hydrostatic skeleton, with the result that fluid is alternately forced into and withdrawn from an inflatable pedal disc. This rhythmic activity forces the disc into the sand.

3. The interval between contractions is approximately 4 minutes at 21° C A pacemaker or set of pacemakers is postulated.

4. Under unusual conditions rhythmic movements may continue for hours. Normally, however, the anemone stops digging within 2 hours. After the pedal disc reaches a hard substratum and sand surrounds the column rhythmic activity ceases.

LITERATURE CITED

ANSELL, A. D., AND E. R. TRUEMAN, 1968. The mechanism of burrowing in the anemone, *Peachia hastata* Gosse. *J. Exp. Mar. Biol. Ecol.*, **2**: 124–134.

BATHAM, E. J., AND C. F. A. PANTIN, 1950a. Muscular and hydrostatic action in the sea anemone, *Metridium senile* (L.). *J. Exp. Biol.*, **27**: 264–289.

BATHAM, E. J., AND C. F. A. PANTIN, 1950b. Inherent activity in the sea anemone *Metridium senile* (L.). *J. Exp. Biol.*, **27**: 290–307.

BATHAM, E. J., AND C. F. A. PANTIN, 1950c. Phases of activity in the sea anemone, *Metridium senile* (L.) and their relation to external stimuli. *J. Exp. Biol.*, **27**: 377–399.

BATHAM, E. J., AND C. F. A. PANTIN, 1951. The organization of the muscular system of *Metridium senile*. *Quart, J. Microscop. Sci.*, **92**: 27–54.

BATHAM, E. J., AND C. F. A. PANTIN, 1954. Slow contraction and its relation to spontaneous activity in the sea anemone *Metridium senile* (L.). *J. Exp. Biol.*, **31**: 84–103.

CARLGREN, O., 1949. A survey of the *Ptychodactiara, Corallimorpharia* and *Actiniaria. Kungl Svenska Vetenskapsakademiens Handlingar, Stockholm,* (4), **1**: 1–121.

CARLGREN, O., 1951. The actinian fauna of the Gulf of California. *Proc. U. S. Nat. Mus.*, **101**: 415–449.

HOYLE, G., 1960. Neuromuscular activity in the swimming sea anemone *Stomphia coccinea* (Muller). *J. Exp. Biol.*, **37**: 671–688.

NEEDLER, M. R., AND D. M. ROSS, 1958. Neuromuscular activity in the sea anemone *Calliactis parasitica* (Couch). *J. Mar. Biol. Ass. U. K.*, **37**: 789–805.

PANTIN, C F. A., 1935. The nerve net of the Actinozoa. I. Facilitation. *J. Exp. Biol.*, **12**: 119–138.

PANTIN, C. F. A., 1952. The elementary nervous system. *Proc. Roy. Soc. London, Series B,* **140**: 147–168.

PANTIN, C. F. A., AND M. VIANNA DIAS, 1952. Excitation phenomena in an actinian (*Bunodactis* sp.) from Guanabara Bay. *Anais da Academia Brasileira de Ciencias,* **24**: 335–349.

PANTIN, C. F. A., 1965. Capabilities of the coelenterate behavior machine. *American Zoologist,* **5**: 581–589.

ROBSON, E. A., 1961. Swimming behavior of the anemone, *Stomphia coccinea. J. Exp. Biol.*, **38**: 343–363.

ROBSON, E. A., 1963. The nerve net of the swimming anemone *Stomphia coccinea. Quart. J. Microscop. Sci.*, **104**: 535–549.

ROSS, D. M., AND L. SUTTON, 1964. Inhibition of the swimming response by food and of nematocyst discharge during swimming in the sea anemone *Stomphia coccinea. J. Exp. Biol.*, **41**: 751–757.

ROSS, D. M., AND L. SUTTON, 1967. The response to molluscan shells of the swimming anemone *Stomphia coccinea* and *Actinostola* (new species). *Can. J. Zool.*, **45**: 895–906.

STEPHENSON, T. A., 1928. *The British Sea Anemones.* The Ray Society, London, 148 pp.

SUND, P. N., 1958. A study of the muscular anatomy and swimming behavior of the sea anemone, *Stomphia coccinea. Quart. J. Microscop. Sci.*, **99**: 401–420.

TRUEMAN, E. R., 1967. The dynamics of burrowing in *Ensis. Proc. Roy. Soc. London, Series B,* **166**: 459–476.

Reference: *Biol. Bull.*, 138: 326–333. (June, 1970).

INACTIVATION OF α-ECDYSONE AND CYASTERONE BY LARVAE OF THE FLESHFLY, *SARCOPHAGA PEREGRINA*, AND PUPAE OF THE SILKWORM, *SAMIA CYNTHIA*[1]

TETSUYA OHTAKI[2] AND CARROLL M. WILLIAMS

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Certain weeds, ferns, and evergreen trees contain complicated sterols closely resembling the insect hormones, α- and β-ecdysones (Fig. 1). When injected into insects, many of these so called "phytoecdysones" have proved to be more active than the insects' own hormones.

A case-in-point is cyasterone, a phytoecdysone which Takemoto, Hikino, Nomoto and Hikino (1967) isolated from the roots of *Cyathula capitata* (Amaranthaceae). As shown in Figure 1, cyasterone is characterized by the presence of a lactone ring at the end of its side-chain. When assayed on mature larvae of the fleshfly, *Sarcophaga peregrina,* cyasterone was twice as active as α-ecdysone (Ohtaki, Milkman and Williams, 1967). In assays on the bee, *Nomia melanderi,* it was 10 times as active (Hsiao and Hsiao, 1969). In assays on pupae of the silkworm, *Samia cynthia,* it was 20 times as active as α-ecdysone and 40 times as active as β-ecdysone (Williams, 1968).

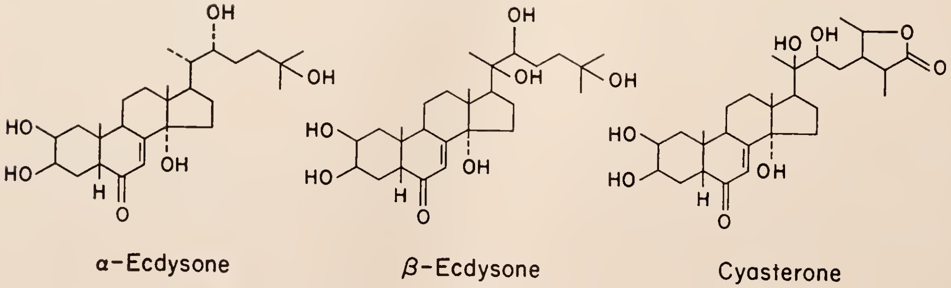


FIGURE 1. Structural formulae of the authentic hormones, α- and β-ecdysones, and the phytoecdysone, cyasterone. The stereochemistry of the side-chain is known only for α-ecdysone.

The greater potency of the hormonal analogue is a phenomenon well known to students of mammalian endocrinology. Especially in the case of the sterol hormones of the gonads and adrenal cortex, amplification of activity has frequently been achieved by chemical modifications of the authentic hormones. The enhanced activity can often be accounted for in terms of the greater resistance of the analogues to enzymes attacking the native hormone (Steelman and Hirschmann, 1967).

---

[1] This study was supported, in part, by NSF Grant No. GB-7963.

[2] Permanent address: Department of Entomology, National Institute of Health, Tokyo, Japan.

In the present investigation we have sought to determine whether this simple explanation suffices in the case of cyasterone. To this end we have studied the decay of hormonal activity in two species of insects injected with cyasterone or α-ecdysone.

MATERIALS AND METHODS

1. Experimental animals

Larvae of *Sarcophaga peregrina* were reared as previously described (Ohtaki, Milkman and Williams, 1968) and utilized as mature larvae or as "standard test abdomens." Additional experiments were performed on diapausing larvae of the silkworm, *Samia cynthia* (weight 2.5 g), which were purchased from dealers and stored at 25° C. One series of experiments made use of pupae of the silkworm, *Hyalophora cecropia* (weight 5 g).

2. Ecdysones

Synthetic α-ecdysone was obtained from Dr. John Siddall, of the Zoecon Corporation. A weighed sample was dissolved in isopropanol and diluted with 9 parts water. The stock solution was further diluted with 10% isopropanol. Parallel experiments were carried out on cyasterone; the latter was obtained from Professor T. Takemoto, of Tohoku University.

3. Injections, extractions, and biological assays

Since all these procedures have already been described in detail (Ohtaki *et al.*, 1968), it suffices to outline the overall plan of the experiments reported here.

Our objective was to measure the "half-inactivation time" of α-ecdysone and cyasterone in fly larvae and silkworm pupae. To this end, each of a series of animals was injected with a certain dose of α-ecdysone or cyasterone. Thereafter at successive intervals, one or more individuals was sacrificed and extracted with a solvent mixture suitable for the recovery of ecdysones. The extract was purified to eliminate toxic water-soluble materials and then subjected to biological assay on standard test abdomens of *Sarcophaga peregrina*. The assay had been calibrated by the injection of known amounts of α-ecdysone or cyasterone. It was therefore possible to equate the endocrine activity of each extract with the activity of a corresponding amount of α-ecdysone or cyasterone. By plotting these values as a function of time, the half-inactivation time was estimated.

The apparent simplicity of these experimental maneuvers is deceptive. For example, we know the identity and amount of hormone injected at zero time; we also know its biological activity as defined by the puparium assay. But during the succeeding hours we know neither the identities nor the amounts of the active materials which we extract, purify, and assay. However, by means of the puparium assay, their combined activity can be determined and equated to that of a certain concentration of α-ecdysone or cyasterone. In short, what we measure is, not the decay of the hormone injected at zero time, but the decay of endocrine activity generated by that injection. As long as the purified extract harvests all materials with ecdysone activity, our measurements are independent of the precise identities of these materials. By suitable tests it was possible to show that this requirement was apparently satisfied in the present study.

RESULTS

1. *Experiments on* Sarcophaga peregrina

The top section of Table I records the per cent of initial activity recovered 0, 1, 3, and 8 hours after the injection of 1 μg α-ecdysone into 0-hour larvae. The hormone in each case was extracted, purified, redissolved in distilled water, and assayed on 10 standard test abdomens. The average puparium index was calculated and converted to micrograms as described by Ohtaki *et al.* (1968). In the right hand column of Table I the recoveries have been corrected for 10% loss in

TABLE I

Decay of ecdysone activity in mature larvae of Sarcophaga peregrina *injected with α-ecdysone or cyasterone*

Substance injected*	Number of larvae injected	Time between injection and extraction (hrs)	% of activity recovered	Corrected % recovery	
α-ecdysone	1	0	88	97	99
	1	0	102	113	
	1	0	78	87	
	1	1	31	34	51
	1	1	53	59	
	1	1	53	59	
	2	3	27	30	23
	2	3	20	22	
	2	3	16	18	
	4	8	1	1	2
	4	8	3	3	
	4	8	1	1	
Cyasterone	1	0	88	97	97
	1	0	92	102	
	1	0	83	92	
	1	1	72	80	78
	1	1	84	93	
	1	1	56	62	
	2	3	46	51	55
	2	3	52	58	
	2	3	28	31	
	2	3	72	80	
	3	8	9	10	13
	3	8	8	9	
	3	8	19	21	

* Each larva received 1 μg hormone in 5 μl of 10% isopropanol.

the extraction and purification procedures. The lower section of Table I summarizes similar experiments performed on *Sarcophaga* larvae injected with 1 μg cyasterone.

In Figure 2 the averaged and corrected recoveries in both experiments have been plotted as a function of the time that elapsed between injection and extraction. Despite the considerable scatter among the individual measurements, it is clear that both hormones are rapidly metabolized by *Sarcophaga* larvae, the half-inactivation time being approximately 1 hour for α-ecdysone and 3–4 hours for cyasterone.

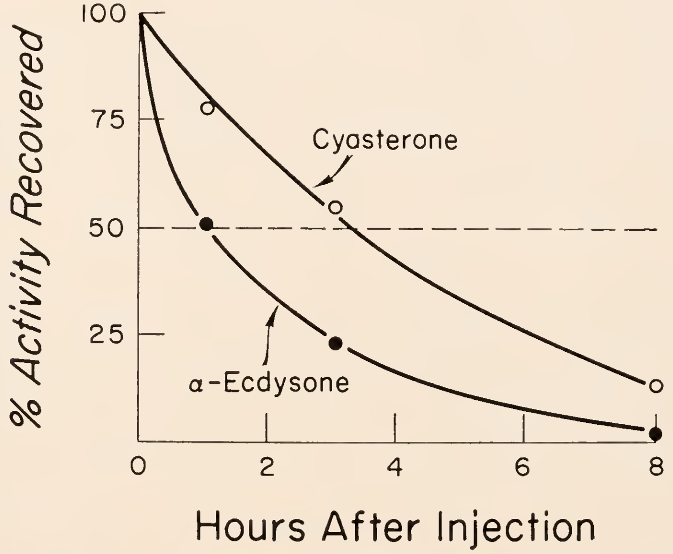


FIGURE 2. The decay of endocrine activity after the injection of 1 μ of cyasterone or α-ecdysone into mature larvae of *Sarcophaga peregrina.*

2. *Experiments on* Samia cynthia

The experiment was repeated on diapausing pupae of the Cynthia silkworm. At zero time each pupa was injected with 5 μg α-ecdysone or cyasterone in 25 μl of 10% isopropanol. The results, corrected for 20% loss of hormone in the extraction of these large insects, are summarized in Table II and Figure 3. Clearly, both materials are much more stable in silkworm pupae than in *Sarcophaga* larvae. Though the results are here again complicated by considerable scatter, the half-inactivation time for α-ecdysone appears to be about 6 hours. In the case of cyasterone, about 60% of initial activity was still present at the 24th hour; by extrapolation we estimate a half-inactivation time of approximately 32 hours.

3. *Experiments on* Hyalophora cecropia

Inactivation of α-ecdysone was also studied on freshly pupated Cecropia silkworms, each of which was injected with 5 μg. As indicated in Figure 3, α-ecdysone was inactivated at the same rate by both species of Lepidoptera.

TABLE II

Decay of ecdysone activity in diapausing pupae of the Cynthia *silkworm injected with α-ecdysone or cyasterone*

Substance injected	Time between injection and extraction* (hrs)	% of activity recovered	Corrected % recovery	
α-ecdysone	0	96	120	102
	0	74	93	
	0	74	93	
	1	64	80	82
	1	80	100	
	1	52	65	
	8	40	50	40
	8	41	51	
	8	16	20	
	24	9	11	18
	24	16	20	
	24	19	24	
Cyasterone	0	68	85	100
	0	88	110	
	0	84	105	
	1	88	110	95
	1	76	95	
	1	64	80	
	8	60	75	85
	8	88	110	
	8	56	70	
	24	40	50	62
	24	72	90	
	24	36	45	

* Each extract was prepared from a single pupa that had been injected with 5 μg of hormone dissolved in 25 μl of 10% isopropanol.

DISCUSSION

1. *Metabolism of α-ecdysone*

In the present and previous studies (Ohtaki *et al.*, 1968) mature larvae of *Sarcophaga peregrina* showed an extremely rapid metabolism of α-ecdysone. Thus, after the injection of 1 μg α-ecdysone, 50% of activity was lost during the first hour and 98% during the first 8 hours. The lower dose of 0.5 μg was metabolized even faster, about 80% of initial activity being lost during the first hour. Inactivation was blocked by low temperatures or anaerobic conditions—a finding that implicates enzymatic attack on the hormone, presumably by one or more "mixed-function oxidases" (Mason, 1965; Kamin and Masters, 1968; Sih, 1969).

These results are therefore in accord with the rapid inactivation of α-ecdysone by the blowfly, *Calliphora erythrocephala*. For example, when α-ecdysone was injected into mature larvae, 80% of activity disappeared in 2 hours (Karlson and Bode, 1969). In similar experiments performed on ligatured abdomens, the half-inactivation time was 3 hours (Shaaya, 1969).

In additional studies of *C. erythrocephala,* Karlson and Bode (1969) extracted from larval fat-body a soluble enzyme system capable of inactivating α-ecdysone *in vitro.* The titer of this system was shown to undergo large and systematic changes during successive phases of metamorphosis.

In experiments performed on *Calliphora vicina,* King and Siddall (1969) observed a much slower inactivation of α-ecdysone than noted in the above mentioned studies of *C. erythrocephala* and *S. peregrina.* For example, when tritiated α-ecdysone was injected into mature larvae, no less than 25% of the tritium label was recovered 12–24 hours later in two materials identified as α- and β-ecdysones. Evidently, the dynamics of ecdysone metabolism can show substantal differences even among closely related species.

In the present investigation a relatively slow inactivation of α-ecdysone was evident in experiments performed on the two species of silkworm pupae. As illustrated in Figure 3, the half-inactivation time was about 6 hours, and 15–20% of initial activity remained after 24 hours.

According to King and Siddall (1969), the first step in the metabolism of α-ecdysone is hydroxylation at C-20 to form β-ecdysone; the experiments in question made use of tritiated α-ecdysone and were performed on *Calliphora vicina* and two species of crustaceans. Subsequently, this finding has been confirmed by Cherbas and Cherbas (1970) in detailed studies of the metabolism of tritiated α-ecdysone by diapausing pupae of the silkworm, *Antheraea polyphemus.* These investigators report, moreover, that the further metabolism of β-ecdysone is accom-

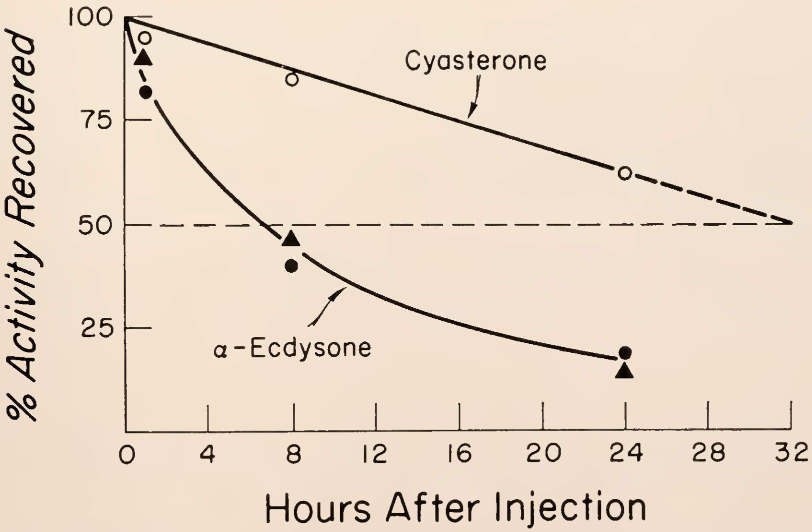


FIGURE 3. The decay of endocrine activity after the injection of 5 μg of cyasterone or α-ecdysone into diapausing pupae of the silkworms, *Samia cynthia* (○,●) or *Hyalophora cecropia* (▲).

panied by the appearance of three materials of increasing polarity but of presently unknown structure or biological activity. Substantially the same results were obtained by Moriyama, King, Nakanishi, Okauchi, Siddall and Hafferl (1970) in a study of the metabolism of tritiated α-ecdysone by mature larvae of the silkworm, *Bombyx mori.*

2. *Comparison of α-ecdysone and cyasterone*

Table III summarizes the parameters of these hormonal materials as ascertained in the present investigation. In the experiments on *Sarcophaga* larvae, cyasterone was twice as active as α-ecdysone and 3–4 times as stable in terms of its half-inactivation time. Therefore, cyasterone's stability can fully account for its greater activity in the fly larvae.

TABLE III

Parameters of two ecdysones in fly larvae and silkworm pupae

	Critical dose* (μg/g live weight)	Activity relative to α-ecdysone	Half-inactivation time (hrs)
Sarcophaga peregrina			
α-ecdysone	0.6	1	1
Cyasterone	0.3	2	3–4
Samia cynthia			
α-ecdysone	2.5	1	6
Cyasterone	0.1	25	32

* Dose provoking a puparium index of 50% in standard test abdomen of *Sarcophaga peregrina*; in the case of Cynthia pupae, the stated dose caused termination of diapause and development of a normal moth.

The bottom half of Table III summarizes the corresponding data for pupae of *Samia cynthia.* Cyasterone proves to be 25 times as active as α-ecdysone but only 5 times as stable in terms of half-inactivation time. So, unlike the situation in *Sarcophaga,* resistance to inactivation can only partially account for the great activity of cyasterone in the lepidopteran. The residual difference must be attributed to other presently unknown properties of the cyasterone molecule which further amplify its activity for silkworm pupae but not for the larval fly.

We are indebted to Professor T. Takemoto of Tohoku University for supplying cyasterone, and to Dr. John Siddall of the Zoecon Corporation for providing synthetic α-ecdysone. We are also grateful to Professor Lynn M. Riddiford and to Lucy and Peter Cherbas for advice and criticism of the manuscript.

SUMMARY

1. The phytoecdysone, cyasterone, is twice as active as authentic α-ecdysone when injected into mature larvae of the fleshfly, *Sarcophaga peregrina.* It is 25 times as active as α-ecdysone when injected into diapausing pupae of the silkworm, *Samia cynthia.*

2. By the biological assay of hormone extracted from injected animals, it was possible to approximate the half-inactivation times of the endocrine activities provoked by the injection of α-ecdysone or cyasterone.

3. In the fly larva the half-inactivation time was about 1 hour for α-ecdysone and about 3–4 hours for cyasterone. Therefore, cyasterone's resistance to inactivation can fully account for its 2-fold greater activity in the larval fly.

4. In diapausing silkworm pupae the half-inactivation time was about 6 hours for α-ecdysone and 32 hours for cyasterone. Consequently, cyasterone's 5-fold greater stability can only partially account for its 25-fold greater activity in the pupal silkworm.

5. The residual difference must be due to other presently unknown properties of the cyasterone molecule which further amplify its activity for silkworm pupae but not for the larval fly.

LITERATURE CITED

CHERBAS, L., AND P. CHERBAS, 1970. Distribution and metabolism of α-ecdysone in pupae of the silkworm *Antherea polyphemus*. *Biol. Bull.*, **138**: 115–128.

HSIAO, C., AND T. H. HSIAO, 1969. Insect hormones: their effects on diapause and development of Hymenoptera. *Life Sci.*, **8**: 767–774.

KAMIN, H., AND B. S. S. MASTERS, 1968. Electron transport in microsomes, pp. 4–26. *In:* E. Hodgson, Ed., *Enzymatic Oxidations of Toxicants*. North Carolina State University.

KARLSON, P., AND C. BODE, 1969. Die inaktivierung des Ecdysons bei der Schmeissfliege *Calliphora erythrocephala* Meigen. *J. Insect Physiol.*, **15**: 111–118.

KING, D. S., AND J. B. SIDDALL, 1969. Conversion of α-ecdysone to β-ecdysone by crustaceans and insects. *Nature*, **221**: 955–956.

MASON, H. S., 1965. Oxidases. *Ann. Rev. Biochem.*, **34**: 595–634.

MORIYAMA, H., D. S. KING, K. NAKANISHI, T. OKAUCHI, J. B. SIDDALL AND W. HAFFERL, 1970. On the origin and metabolic fate of α-ecdysone in insects. *Gen. Comp. Endocrinol.*, in press.

OHTAKI, T., R. D. MILKMAN AND C. M. WILLIANS, 1967. Ecdysone and ecdysone-analogues: their assay on the fleshfly *Sarcophaga peregrina*. *Proc. Nat. Acad. Sci.*, **58**: 981–984.

OKTAKI, T., R. D. MILKMAN AND C. M. WILLIAMS, 1968. Dynamics of ecdysone secretion and action in the fleshfly *Sarcophaga peregrina*. *Biol. Bull.*, **135**: 322–334.

SHAAYA, E., 1969. Der Ecdysontiter während der Insektenentwicklung. VI. Untersuchungen über die Verteilung des Ecdysons in verschiedenen Geweben von *Calliphora erythrocephala* und über seine biologische Halbertszeit. *Z. Naturforsch.*, **24**: 718–721.

SIH, C. J., 1969. Enzymatic mechanism of steroid hydroxylation. *Science*, **163**: 1297–1300.

STEELMAN, S. L., AND R. HIRSCHMANN, 1967. Synthetic analogs of the adrenal cortical steroids, pp. 345–389. *In:* A. B. Eisenstein, Ed., *The Adrenal Cortex*, Little, Brown and Co.

TAKEMOTO, T., Y. HIKINO, K. NOMOTO AND H. HIKINO, 1967. Structure of cyasterone, a novel C_{29} insect-moulting substance from *Cyathula capitata*. *Tetrahedron Lett.*, 3191–3194.

WILLIAMS, C. M., 1968. Ecdysone and ecdysone-analogues: their assay and action on diapausing pupae of the Cynthia silkworm. *Biol. Bull.*, **134**: 344–355.

Reference: *Biol. Bull.,* **138**: 334–343. (June, 1970).

ISOLATION, LOCALIZATION AND BIOSYNTHESIS OF CRASSIN ACETATE IN *PSEUDOPLEXAURA POROSA* (HOUTTUYN)[1]

JOHN R. RICE, CONSTANTIN PAPASTEPHANOU AND DAVID G. ANDERSON

Department of Biochemistry, University of Miami, School of Medicine, Miami, Florida 33152

Crassin acetate was first isolated by Ciereszko, Sifford and Weinheimer (1960), from the whole dried gorgonian *Pseudoplexaura porosa* (Houttuyn). Ciereszko (1962), later reported that this diterpene lactone was present in very high concentration in the symbiotic zooxanthellae isolated from this gorgonian. We have been studying sesquiterpene hydrocarbon biosynthesis in zooxanthellae isolated from *P. porosa.* A major difficulty in isolating pure zooxanthellae is in separating them from a large volume of colorless micro-crystals. The objectives of the present study were to check the identity of the crystals with crassin acetate, to eliminate the possibility that their extracellular occurrence is an artifact of the isolation procedure, to determine the histological relationship of the crystals, the zooxanthellae, and the host tissue, and to study the metabolism of crystalline crassin acetate.

MATERIALS AND METHODS

Isolation of crassin acetate crystals from Pseudoplexaura porosa

P. porosa was collected from the coral patch reefs known as Bache Shoal just off Elliott Key, Florida, transported in sea water, and processed immediately on return to land.

Segments of gorgonian 3 to 4 cm long were homogenized in a Waring Blendor at full speed for 10 seconds with approximately twice their volume of sterile 50 per cent sea water, a medium which was used throughout this preparation. The medium was sterilized by passage through a 0.45 μ pore size Millipore filter. Large fragments and gorgonian stipes were removed by a single passage through cheese cloth. After the strained homogenate was centrifuged at 3000 g in 250 ml centrifuge bottles for 15 minutes, the supernatant was discarded and the buff-colored surface layer was removed by gentle swirling with medium. The remainder of the pellet was resuspended in a 20-fold volume of medium and centrifugation and washing was repeated two more times.

The final pellet was resuspended in six times its volume of medium, immediately transferred to 12 ml centrifuge tubes and centrifuged at 2000 g in a swing head for ten minutes. The four layered pellet which formed was separated into fractions by differential resuspension using a syringe-controlled pipette. In general, it was technically simple to resuspend the bulk of a layer without introducing substantial contamination from the layer immediately below. The shallow

---

[1] Work supported by research grant GM-12229 from the National Institute of General Medical Science, Public Health Service.

top layer (debris) was resuspended by a gentle stream and discarded. Progressively stronger streams from the pipette served to resuspend the second (zooxanthellae) layer and the third (crystalline) layer. Fractions from these two layers were reserved as a predominantly zooxanthellar fraction, a mixed fraction, and a crystalline fraction. The fourth (spicule) layer was discarded. The reserved fractions were repeatedly processed via centrifugation and differential resuspension until the zooxanthellae and crystals were minimally contaminated with each other.

Small scale extraction of crassin acetate (radiochemical experiments)

Fresh *P. porosa,* about 25 grams, was cut into 3 cm lengths and extracted three times in a Waring Blendor with a total of 250 ml of 50% petroleum ether in methanol. Two grams of Hyflo-SuperCel were added as a filter aid for the first extraction. The mixture was filtered under vacuum through Whatman No. 1, and the filter cake was re-extracted in the Blendor. The fourth and final extraction employed 50 ml of methanol. The combined extracts formed two layers, the epiphase petroleum ether and the hypophase approximately 80% methanol in H_2O. After phase separation the petroleum ether phase was washed twice with 80% methanol in H_2O, and the methanol phase with petroleum ether. Each wash was added to its corresponding fraction. Concentration of the methanol rich fraction gave an oily residue. Methanol washes of the residue were combined with the soluble methanol fraction and decolorization achieved by boiling with acid-washed charcoal and filtering. Addition of an equal volume of warm water followed by cooling crystallizes crassin acetate. Recrystallizations and further decolorizations, if required, proceeded via the above procedure using a minimal amount of methanol as solvent.

Large scale extraction of crassin acetate

Colonies of *P. Porosa* were dried in the open air. When constant weight was achieved, the cortex was stripped from the stipe and reduced to a coarse powder in a Waring Blendor. Five pounds of the dried powder were placed in the continuous extractor described by Ciereszko (1966) and subjected to continuous extraction with petroleum ether so as to remove the bulk of pigments and some lipids that complicate the crystallization of crassin acetate. When the extractant overflow was barely tinted, extraction was interrupted, the extracted cortex was dried at room temperature and then subjected to a single methanol extraction by a batch process, 500 ml per 100 grams of dried cortex.

While by no means colorless, this extract produced purified crassin acetate crystals when the volume was reduced and water added to incipient crystallization, followed by cooling. Decolorization with charcoal and recrystallization from methanol-water resulted in pure crassin acetate, melting point 141° C.

Infrared spectroscopy

For infrared spectroscopy a portion of the crystals isolated in unmodified form by homogenization in 50 per cent sea water and centrifugation were washed essentially free of the few remaining zooxanthellae on a coarse sintered-glass filter and then compressed in a KBr pellet. The spectrum was taken on a Beckman IR-8 infrared spectrophotometer.

Histological preparation

Fresh segments of gorgonian were relaxed for 2 to 5 minutes in 1.2% $MgCl_2$, fixed for 12 hours in 10% acetate-buffered formalin, pH 6.5–8.0, and decalcified for 12 hours in 0.5 *M* sodium ethylenediaminetetraacetate (EDTA), pH 8.3. Specimens were embedded in 6% gelatin and frozen sections cut at 10 μ on a CO_2 freezing microtome. Some sections were stained by flotation in 0.1% toluidine blue for 15 seconds and briefly washed in water. Other sections were not stained. All sections were permanently mounted using Zwemer's glycrogel mounting medium.

Acid treatment of the cortex

Four-inch segments of gorgonian tip were dried at room conditions to constant weight. Each segment was exposed to 10 ml of 2 *N* HCl for three days, after which the remaining solid material was washed with distilled water on weighed filter paper. The filter paper and gorgonian remains were dried for three days under room conditions and weighed.

Chromatography of crassin acetate

Thin layer chromatography employed aluminum oxide and silica gel. Precoated plates obtained from Brinkmann Instruments, Westbury, New York, consisted of aluminum oxide (Type E) with fluorescent indicator F_{254}, Merck AG 5751/0025, and silica gel with F_{254}, Merck AG 5714. A detection spray (Attaway, Wolford and Edwards, 1965) of 5% vanillin in concentrated H_2SO_4 gives a gray-violet spot with crassin acetate on heating at 110° C for 10 minutes. With this detection technique, thin layer plates with F_{254} provide a whiter and more uniform background than plates lacking the indicator. Silica gel plates were run with 20% acetone in benzene. The R_f of crassin acetate in this system is 0.49. Twenty per cent dioxane in benzene on aluminum oxide gave a crassin acetate R_f of 0.59. Aluminum oxide plates with acetone:benzene (1:4) gave an R_f of 0.75.

In column chromatography of crassin acetate, an aluminum oxide (Merck for chromatography) column 1.4 × 11 cm was developed with stepwise increases of ethyl acetate in petroleum ether. Crassin acetate is eluted by 70% ethyl acetate.

Colorimetric determination of crassin acetate

In a direct method, crassin acetate in methanol was allowed to stand at room temperature with 3 ml of 5% freshly prepared vanillin in conc. H_2SO_4. The product has an absorption maximum at 540 mμ and was read at this wave length against a reagent blank containing the standard volume of methanol. Absorbance is linear within the range of 10 to 50 μgrams of crassin acetate. A disadvantage of this method arises from the high blank caused by methanol. A second colorimetric procedure below avoids this difficulty.

Colored spots on the developed and sprayed thin layer chromatogram were scraped off and eluted with small multiple volumes of acetone, adjusted to 1.0 ml and read at 540 mμ. Standardization was accomplished by the same technique. While results are somewhat erratic and routine standardization is required, this technique is useful in the range of 20 to 200 μgrams of crassin acetate.

Incubation with radioactive substrates

Radioactive carbonate in the form of $Na_2{}^{14}CO_3$, 6.4 μc/μmole was purchased from New England Nuclear, and $^{14}CH_3COOH$, 51 μc/μmole as the sodium salt was purchased from Amersham-Searle. Incubations were performed in stoppered Pyrex 500 ml reagent bottles. The radioisotope in 0.2 ml of 0.05 *M* KOH was placed in the bottles on land and taken to the reef area. To each bottle 400 ml of sea water from the collection site was added. A single colony of *P. porosa* was collected and a 10 cm segment cut from each tip. Seven such segments were placed in each sea water-isotope solution, sealed, and exposed to direct sunlight for a period of two hours. The gorgonian segments were then processed for isolation of crassin acetate. These seven gorgonian segments represent about 25 grams wet weight and seven grams dry weight. Two experiments were carried out using two different colonies. In experiment I the specific radioactivity of the carbonate ^{14}C was 0.11 μc/μmole and that of acetate 2-^{14}C was 0.20 μc/μmole; the total radioactivity was 125 μc/liter and 10 μc/liter, respectively. In experiment II the specific radioactivity of the carbonate ^{14}C was 0.165 μc/μmole and that of acetate 2-^{14}C was 50 μc/μmole; the total radioactivity was 182 μc/liter and 625 μc/liter, respectively. The value for $CO_3{}^{2-}$ and $HCO_3{}^-$ used in calculating specific activity was 2.31 milliequivalent/liter of sea water.

Radioactivity measurements

Scintillation methods were employed for radioactivity measurements. Two instruments, a Packard Tricarb Liquid Scintillation Spectrometer Model 4312 and a Beckman Liquid Scintillation System Model LS were employed. In both instruments, windows which counted ^{14}C with about 75% efficiency were used. Efficiency was routinely checked with internal standards dependent on the scintillation medium. Toluene with PPO and POPOP served in counting toluene-miscible samples, while naphthalene-dioxane, PPO and POPOP was used for aqueous samples. Suspected quench was detected and corrected for by use of internal standards.

Determination of labeling pattern, acetate vs. *crassin*

Distribution of radioactivity between the crassin ring and the acetate moiety of crassin acetate is determined by acid hydrolysis of the ester, separation of acetic acid and crassin by lyophilization in a closed system, and finally, counting the residual crassin and products derived therefrom and the distillate, acetic acid. Hydrolysis of a weighed sample of crassin acetate of constant specific radioactivity is carried out with 2 *N* HCl in a sealed ampoule at 110° C for 17 hours. The ampoule is transferred to one arm of a U-tube and broken. This arm is then chilled by immersing in solid CO_2-acetone. The system is evacuated to a pressure of 0.1 mm Hg and then sealed. The second arm of the U-tube is placed in the cold bath and the contents of the other arm allowed to warm to room temperature. All volatile components of the hydrolyzate will thus be transferred to the second arm while the non-volatile components remain behind.

Results and Discussion

Infrared absorption studies

Comparison of the infrared absorption spectrum of the unmodified crystals isolated from *P. porosa* with the spectrum of authentic crassin acetate (see Fig. 1) reveals that the spectra are essentially identical. Furthermore, it is evident that the unmodified crystals isolated directly from the homogenate of *P. porosa* in sea water are crassin acetate of a high degree of purity. Since the crystal fraction contains some visible contamination, recrystallization is necessary to obtain an adequate melting point. One recrystallization is usually sufficient to give material with a constant melting point of 140–141° C. This compares with the melting point of 138–140° C reported by Weinheimer, Schmitz and Ciereszko (1968), who also showed that crassin acetate is based on the 14 carbon cembrane ring (Fig. 2).

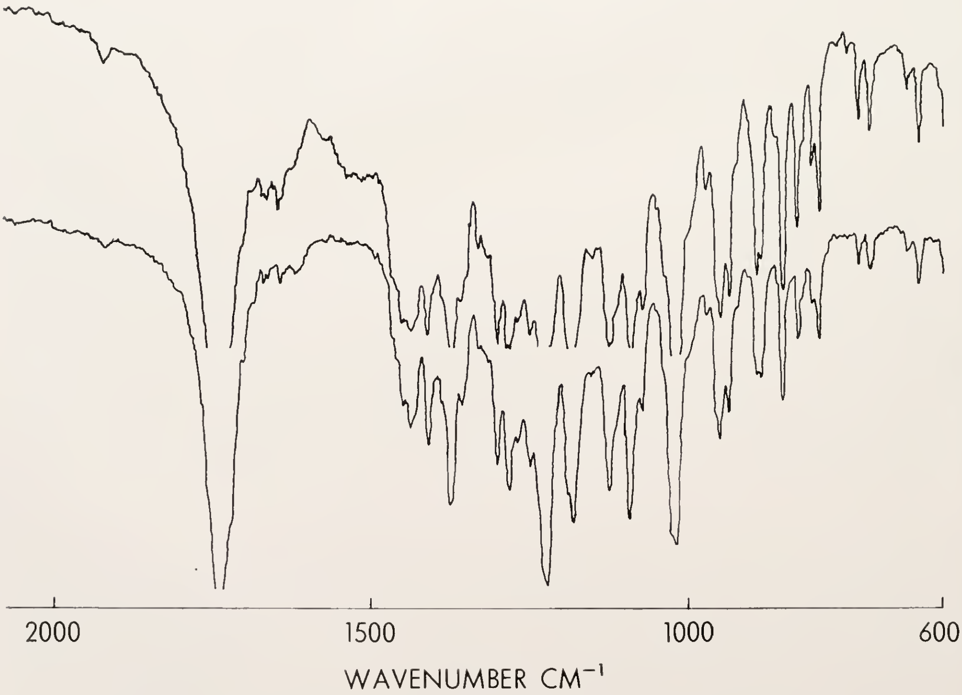


Figure 1. Infra red spectra; isolated unmodified crystals (upper curve) compared with authentic crassin acetate (lower curve).

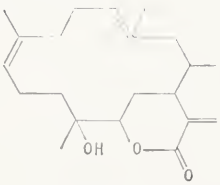


FIGURE 2. Structure of crassin acetate, Weinheimer *et al.* (1968).

Histological studies

Ciereszko (1962), suggested that crassin acetate is produced by and contained within the symbiotic zooxanthellae of the gorgonian. The occurrence of free crystals of crassin acetate might, therefore, be an artifact of the isolation process. Cover slip crush preparations of individual polyps of *P. porosa* readily demonstrated that the crystals identified as crassin acetate are indeed extracellular to the zooxanthellae. However, sectioning of fixed material was necessary to establish precise anatomical relationships. Most standard fixation and embedding techniques dissolve crassin acetate, hence the procedure detailed in the materials and methods section was adopted. Even then, an absolute minimum of fixation and decalcification time was necessary. Crassin acetate is slowly soluble in buffered formalin and EDTA probably by conversion of the lactone to the water soluble carboxylate.

Toluidine blue stains zooxanthellae and the stipe of the gorgonian a deep green. The remainder of the gorgonian tissues stain varying intensities of blue with the exception of certain cell types, particularly those lining the stomodeum of the polyps, which stain magenta. Crystals of crassin acetate and the organic matrix of the calcium carbonate spicules of the gorgonian do not stain. A typical photomicrograph of an unstained section (see Fig. 3), shows the relationship of host tissue, zooxanthellae and crystals of crassin acetate. Examination of the photomicrograph indicates a close association of crassin acetate crystals with the symbiotic zooxanthellae found in the host endoderm. Although the crystals may be found free in the cavities and longitudinal canals of the polyps, they are found in greatest profusion embedded in host tissues which contain zooxanthellae. Furthermore, crystals are absent from zooxanthellae-free tissue. This leads us to conclude that crassin acetate is most likely produced by zooxanthellae, or by the interaction of the host endoderm-zooxanthellae complex.

Intracolonial distribution of crassin acetate

Studies of the distribution of crassin acetate along the length of the colony from base to tip employing the semiquantitative thin-layer technique and the direct colorimetric measure reveal no striking variations in the concentration of crassin acetate in the gorgonian cortex. This suggests a rapid synthesis followed by quiescence rather than continued slow synthesis. An alternative explanation would be turnover of the crassin acetate, a possibility which we are further exploring. However, the cortex represents larger increments of the total colony with progress along the stipe from base to tip. Thus considering the whole colony including the stipe, the concentration of crassin acetate increases from base to tip.

Concentration of crassin acetate

The concentration of crassin acetate in the *P. porosa* cortex was estimated by two methods: (1) the semi-quantitative thin-layer technique and (2) weighing the crassin acetate purified and isolated from a known weight of dried cortex. Both methods gave similar results. Averaging six determinations on three samples shows 4.4% of the dry cortex and 1% of the whole dried colony as the minimum concentration of crassin acetate. No attempt was made to estimate losses which were probably large.

A substantial portion of the cortex is made up of calcium carbonate spicules. Therefore we used dilute HCl treatment to dissolve the spicules and found that 39 to 41% of the dried tip cortex (with or without the central stipe) was not soluble in acid. Neglecting the possibility that acid treatment solubilized substantial amounts of material in addition to calcium carbonate, we can conclude that crassin acetate represents at least 10% of the organic substance (acid insoluble) of the dried gorgonian cortex.

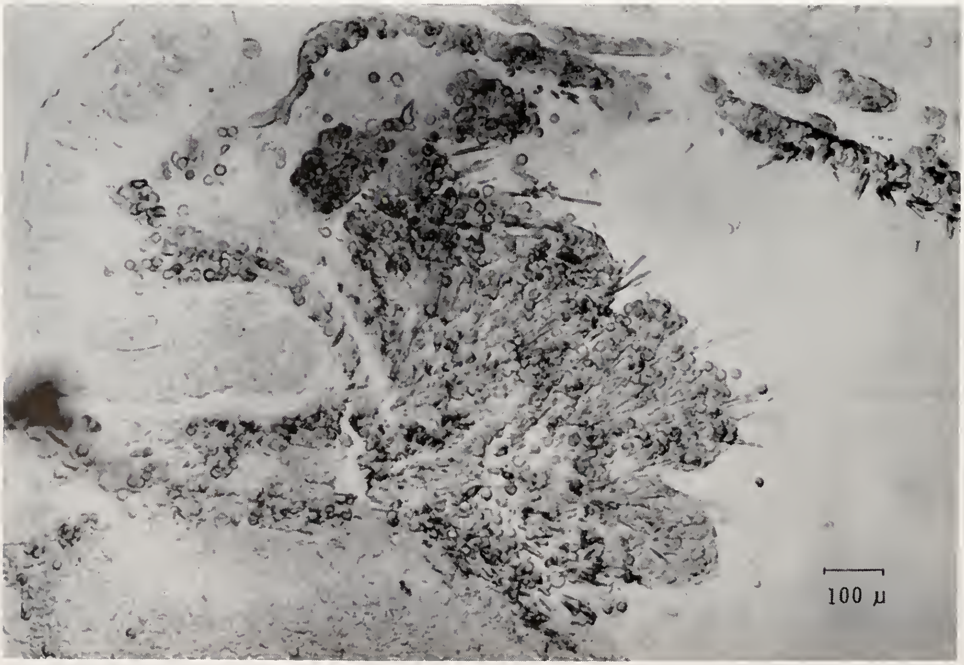


FIGURE 3. Unstained decalcified frozen section of *P. porosa* polyp. Elongate forms are crassin acetate crystals, many project into the digestive cavity. Darker circular bodies are zooxanthellae located primarily in host gorgonian endoderm. Paler tissues which are zooxanthellae free are largely host exoderm.

Metalobism of crassin acetate

When intact tips of *P. porosa* colonies are incubated in sea water with ^{14}C carbonate or acetate, a substantial portion of the label is incorporated into the organic material of the gorgonian-zooxanthellae complex. However, net incorporation into crassin acetate is small. From Table I it is apparent that crystallization of the isolated crassin acetate quickly achieves constant specific radioactivity in spite of the wide range of radioactive products which must have formed. It is also apparent that acetate is superior to carbonate as a crassin acetate precursor by approximately ten-fold.

TABLE I

*Labeling of crassin acetate in cpm/μmole**

Crystallization Number	Carbonate ^{14}C		Acetate 2-^{14}C	
	Experiment I	Experiment II	Experiment I	Experiment II
1	4.5	—	—	—
2	4.1	—	43.2	—
3	3.2	1.1	47.3	925
4	3.7	1.2	46.2	1010
9	3.3	—	—	—

* In experiment I, tips from a single gorgonian colony were used. In experiment II, a different single colony served as a source of tips. See "Materials and Methods" for details.

In some labeling experiments high specific activity precursors were employed. In addition carbonate and acetate enter into a host of pathways. Because specific coprecipitation might account for the swift attainment of constant specific radioactivity a further test system employed column chromatography. Fractions were collected over the course of elution, evaporated, weighed and the radioactivity of the total sample determined. Results are presented in Table II.

Except for the first fraction, satisfactory specific radioactivity is maintained across the peak. We conclude that crassin acetate has been labeled in these experiments.

Incorporation of acetate radioactivity into crassin acetate might merely reflect the exchangeability of the acetate ester moiety. Release by hydrolysis and trapping

TABLE II

*Distribution of radioactivity in crassin acetate fraction**

Fraction	Wt. of crassin acetate	cpm	Specific radioactivity cpm/mg
26	2.7	175	64
27	11.5	1276	110
28	19.0	2374	124
29	7.3	846	122
30	2.1	214	102

* See "Materials and Methods" for details. Crassin acetate applied to the column had an activity of 122 cpm/mg.

of the ester acetate revealed that a substantial portion, but less than half, of the label in crassin acetate was present in the acetate ester moiety. Two experiments gave essentially identical results; 960 cpm in released acetic acid from 2480 cpm in the original crassin acetate, and 872 cpm in acetic acid from 2010 cpm in the original crassin acetate, 38.6 and 43.4% of the label in the acetate moiety, respectively. We can conclude that a substantial portion of the label in crassin acetate arises by ester exchange, or esterification of newly formed crassin. The crassin ring is also labeled, however, since a large portion of the label is found in the residue remaining after hydrolysis. Further experiments to define the labeling pattern in crassin acetate, specifically, the crassin ring, are in progress.

These experiments have shown that the gorgonian-zooxanthellar complex has the capability of synthesizing crassin acetate *de novo*. The possibility existed that crassin acetate was accumulated or modified, and segregated by feeding and digestion processes. This is obviously not the case. Our various attempts to demonstrate biosynthesis of crassin acetate by isolated *P. porosa* zooxanthellae were unsuccessful. Thus, in conjunction with the present results, our hypothesis that crassin acetate is formed as a joint venture by gorgonian and zooxanthellae is still tenable.

The extremely high level of crassin acetate found in *P. porosa* accentuates the problem of function of this diterpene. Ciereszko *et al.* (1960), have found that crassin acetate is highly toxic to a number of marine vertebrates, as well as being antiprotozoal and antibacterial; they, therefore, conclude that crassin acetate may have a protective function. Most personnel in our laboratory who have worked with *P. porosa* homogenates have experienced a toxic reaction about the eyelids resembling a pruritic contact eczematous dermatitis. The untested assumption is that the reaction is caused by microcrystalline crassin acetate.

Aside from toxicity no function of crassin acetate has been demonstrated. It is tempting to speculate that it or other terpenes of zooxanthellae may serve as metabolic energy stores for the zooxanthellae or the gorgonian host.

We wish to thank Drs. A. J. Weinheimer, Leon S. Ciereszko and Tom K. B. Karns, Department of Chemistry, The University of Oklahoma, for providing the infrared absorption spectra as well as results of structural studies prior to publication; Dr. Frederick M. Bayer of the Rosenstiel School of Atmospheric and Marine Science, University of Miami, for aid in gorgonian identification; and Dr. Irwin Berman and Mrs. Dorothy Stokes of the Department of Anatomy for aid and advice in the histological studies. Requests for reprints should be addressed to Dr. David G. Anderson.

Summary

1. The particulate portion of a homogenate of the gorgonian *Pseudoplexaura porosa* (Houttuyn) can be separated into fractions by repeated centrifugation and differential resuspension.

2. One of these fractions is crystalline and has been identified as crassin acetate, a diterpene lactone, by the infrared absorption of the original crystals and by melting point determination after recrystallization.

3. Histological sections demonstrate that crassin acetate crystals are extracellular, in part, to the gorgonian tissues, and *in toto*, to the symbiotic zooxanthellae of this organism. However, the crystals occur in association with gorgonian endoderm containing zooxanthellae, thus suggesting that crassin acetate is a product of host-zooxanthellae interaction.

4. Quantitative determinations of crassin acetate show a minimum concentration of 10% of the organic dry weight of the cortex. It was also found that cortex from both youngest and oldest parts of the gorgonian colony contain essentially equal concentrations of crassin acetate.

5. Both CO_2 and acetate are precursors of the diterpene, acetate being about ten times more efficient.

LITERATURE CITED

ATTAWAY, J. A., R. W. WOLFORD AND G. I. EDWARDS, 1965. Determination of esters by thin layer chromatography. *Anal. Chem.*, **37**: 74–76.

CIERESZKO, L. S., D. H. SIFFORD AND A. J. WEINHEIMER, 1960. Chemistry of Coelenterates, I. Occurrence of terpenoid compounds in Gorgonians. *Ann. New York Acad. Sci.*, **90**: 917–919.

CIERESZKO, L. S., 1962. Chemistry of Coelenterates. III. Occurrence of antimicrobial terpenoid compounds in the zooxanthellae of alcyonarians. *Trans. New York Acad Sci.*, **24**: 502–503.

CIERESZKO, L. S., 1966. A versatile continuous percolator-extractor. *J. Chem. Education*, **43**: 252.

WEINHEIMER, A. J., F. J. SCHMITZ AND L. S. CIERESZKO, 1968. Chemistry of coelenterates—VII, pp. 135–140. *In:* H. D. Freudenthal, Ed., *Drugs from the Sea.* Marine Technology Society, Washington, D. C.

Reference: *Biol. Bull.*, **138**: 344–353. (June, 1970).

RESTORATION OF REGENERATIVE ABILITY IN LIGATED STEMS OF *TUBULARIA* IN AN ELECTRIC FIELD[1]

S. MERYL ROSE

Department of Anatomy, Tulane University School of Medicine, Riverside Research Laboratories, Belle Chasse, Louisiana 70037

There is evidence that the locus of regeneration and its direction may be controlled by the bioelectric field. Mathews (1903) measured potential differences along stems of hydroids and between hydranths and stems. The more apical (distal) regions were electronegative to more basal (proximal) regions at the surface. A gradient in potential, with the distal surface region more negative in most species, has been reported (Hyman, 1920; Hyman and Bellamy, 1922; Child and Hyman, 1926; Barth, 1934b). Two species, *Obelia commissuralis* (Lund, 1925) and *Eudendrium* sp. (Barth, 1934b) were found to be more positive distally.

Mathews (1903) suggested that the electrical polarity might determine the direction of differentiation. He also mentioned a preliminary experiment in which it appeared that an imposed current inhibited regeneration in one direction but not in the opposite direction. It was clearly demonstrated by Lund (1921) that an imposed direct current field can determine the polarity of regeneration in *Obelia*. Ordinarily pieces of stem of *Obelia* form hydranths at both ends. In fields of appropriate strength only that end, either distal or proximal, which faced the positive pole regenerated. Lund (1925) believed that regeneration was blocked at an end because the sign of the applied field was opposed to the sign of the charge across the coenosarc at that end. Barth (1934a) studied the effect of applied fields on *Tubularia crocea, Eudendrium* sp., and *Pennaria* sp. *Eudendrium*, like *Obelia commissuralis*, the species used by Lund, is positive at distal surfaces with respect to more proximal surface regions. *Tubularia crocea* and *Pennaria* on the other hand are more negative in their distal regions (Barth, 1934b). In all four of the forms the polarity of regeneration was reversed when the sign of the applied field was opposed to the sign inherent in the stems. This made it appear that the inherent field might determine morphogenetic polarity as does the applied field.

Marsh and Beams (1952) demonstrated that the morphogenetic polarity of pieces of planaria can also be reversed by an applied field. At low field strengths heads appeared at anterior ends only. In fields of intermediate strength heads formed at both ends. With stronger fields there was complete reversal of polarity and heads formed only at the original posterior ends. In planaria as in the hydroids it seemed that the electric field might determine the direction in which controlling information might pass from cell to cell. This as a possible mechanism was first suggested by Lund (1925).

---

[1] This work has been supported by grant G23866 from the National Science Foundation.

In the course of some grafting experiments in *Tubularia* it was learned that morphogenetic control is polarized (Rose, 1957). If graft and host were combined with the same polarity and the graft was in the distal position the graft suppressed the formation of like structures in the host. The host was still capable of making all regions more proximal than that being formed by the graft. When the graft and host were combined distal to distal with opposed polarity neither affected the other. Although tightly joined both formed distal structures. The repressive information appeared to move from distal to proximal only. The region of contact was like a watershed from which movement was away from the point of contact and not across it. The same relationship holds for *Stentor* (Tartar, 1964). An anterior region could prevent a potential anterior region from forming anterior structures if they were aligned with the same polarity. When combined anterior to anterior neither affected the other.

If, as some of the earlier investigators suspected, the polarity of differentiation were determined by the electrical polarity of the stem, one should be able to block the effect of graft on host by a field of appropriate strength and sign. It was learned that the effect of a graft on its host could be blocked by an applied field when the graft faced the negative pole (Rose, 1963).

Other experiments with *Tubularia* indicated that regional differentiation might be controlled by positively charged repressors moving in the bioelectric field (Rose, 1966, 1967ab). Positively charged regionally specific repressors were collected. These prevented differentiation of the level from which they were extracted and all more distal levels. For example, positively charged materials separated by electrophoresis from the middle region of the hydranth primordia prevented younger primordia from forming middle and distal structures but allowed all more proximal levels to develop. It appeared that differentiation proceeds because positively charged repressors from the most distal region are carried away from their locus of origin toward more negative proximal regions. The cells receiving the distal repressors do not form the most distal structures but do become the most distal structures from which they are not receiving repressors. The cells at the second level, as they differentiate, also release repressors which move proximally along the electrical gradient. Second level repressors prevent the formation of the second and first levels behind them but do not repress differentiation of the third level. As each level in its turn develops into the most distal structures not forming distal to it, differentiation proceeds until all levels have formed along the polarized electrical gradient.

If an artificial field of sufficient strength is applied to the combination of a distal graft attached to the distal end of a whole primordium the control of the graft is blocked when the combination faces the negative pole. In this case the applied field opposes the bioelectrical polarity and would tend to block the movement of positively charged molecules from graft to host (Rose, 1963).

If the theory of control by charged repressors is correct, one would expect a bioelectric field of a certain strength to be necessary for regeneration. Further, one should be able to weaken the bioelectric field and thereby prevent regeneration. The plan in the work to be described was to find a method of decreasing the normal bioelectric field to a level at which regeneration did not occur. After this was done stems were placed in a field of a strength already known to change polarity in some cases. If they were then able to regenerate new structures after they had been given a new field, it would appear that a bioelectric field of a certain strength is necessary for regeneration in *Tubularia*.

Materials and Methods

The *Tubularia* most used in these studies was the common species found in the harbor at Woods Hole. This was formerly thought to be *Tubularia crocea* but is now known to fit the description for *Tubularia spectabilis* (Miller, 1969). *Tubularia spectabilis* from the Cape Cod Canal and *Tubularia crocea* from New Bedford were also used.

In all of this work it was important to know the original polarity of the regenerating stems. For this reason when stems were cut away from colonies, the distal end was cut away with a cut at right angles to the main axis of the stem. The proximal end was always cut obliquely. Whenever stems were ligated, distal and proximal ends and which way the stems faced in a field were indicated by coded colored ligatures.

When stems were subjected to an electric field they were held in place in grooves in slabs of 2 per cent agar in sea water. They were held in place by a covering layer of cotton organdie. This allowed pasteurized sea water dropping from capillary siphons to flow over and around the stems without dislodging them. The dripping directed to the region where stems were confined was at the rate of a liter in 6 to 8 hours.

The agar slabs were made and used in plastic trays measuring 17 × 8.5 × 0.8 cm. The field was supplied by a Vokam power supply type 2541. The current density was maintained at 26 microamperes per square millimeter. The drop in potential was 0.3 to 0.4 volt per cm along the agar trays. The temperature was maintained at 14–15° C.

Measuring electrodes were silver-silver chloride mounted in glass tubes. Contact with the stems was by way of electrode tips of cotton umbilical tape impregnated with 2 per cent agar and moistened with sea water. Only electrodes whose inherent potential differences were less than 0.2 mv were used. Potential differences were measured with a Hewlett-Packard microvolt meter, model 425A. Stems to be measured were quickly dried on filter paper and placed across the cotton ends of the electrodes. Great care was exercised not to dry the perisarc too much. If this happened ends were injured, coenosarc shrank, air bubbles appeared and the injured regions became negative to nearby uninjured regions by approximately 4 mv. Such stems were discarded. It is much easier to measure the potential differences of ligated stems without injuring the stems. The ligatures act as purse strings closing the protective secreted perisarc over the living coenosarc.

Experimental Section

Potential differences were measured in 200 stems before and during transformation of distal ends to hydranths. It was confirmed that in general in *Tubularia* there is a graded potential difference with distal regions being more negative (Barth, 1934b). It was also confirmed that potential differences vary appreciably and proximal regions may be more negative especially in the vicinity of a branch (Hyman, 1920).

Pieces of stem 10–15 mm long were removed from young colonies usually within two hours of the time they were collected and always within 24 hours. Both the colonies and the stems removed from them were kept in running sea water until they were measured in air as described in Materials and Methods. Potential differences were measured along the perisarc between the distal end and the middle and between the proximal end and the middle of the isolated stems. A typical experiment is recorded in Table I. At the left of the table are the readings in millivolts of the potential differences at the two ends with reference to the middle. In this experiment no measurements were made before 12 hours after the stems had been removed from the colony. In other experiments when stems were measured from 5 to 12 hours after isolation some had positive ends. This transient positivity had also been observed before but was usually over in from one to two hours (Barth, 1934b).

By 12 to 17 hours after isolation all of the distal ends had become negative by 0.9 to 1.5 mv. Most of the proximal ends had lower negative values at the twelfth and thirteenth hours, ranging from −0.3 to −1.0 mv. In the next four hours the highest negative value at a proximal end was 0.1 mv and the other four proximal ends had become positive. All of the stems formed hydranths at the distal end and none did at the proximal end. At the time of measurement three of the stems were showing the first signs of a primordium. The distal end and a region behind the future primordium had become more translucent. These three had readings of −1.2, −1.3 and −1.5 mv. The others were within a few hours of

TABLE I

Potential differences in millivolts between ends and middles in stems with open and with ligated ends

Hours	Open at both ends		Hours	Open at proximal end		Hours	Ligated at both ends		Hours	Open at distal end	
	D-M	P-M		D-M	P-M		D-M	P-M		D-M	P-M
12	−0.9	−0.6	12	−0.9	*−0.9*	12	−1.2	−1.2	12	−1.3	−0.7
12	−1.2	−1.0	12	−1.4	−0.8	12	−1.0	−0.4	13	−0.8	+0.3
12	−1.0	−0.8	12	−1.0	*−0.7*	12	−0.3	−0.1	13	−0.9	+0.6
13	−0.9	−0.8	13	−0.5	−0.3	13	−0.8	−0.2	14	−1.4	−0.4
13	−1.2	−0.3	13	+0.7	*−1.1*	13	+0.1	−0.4	14	−1.4	0.0
14	−1.5	+0.2	14	+0.1	*−1.2*	14	−0.4	+0.6	17	−1.5	+0.2
14	−1.5	+0.3	17	+0.6	*−0.9*	14	+0.1	+0.1	17	−1.6	+0.7
17	−1.3	+0.6	17	−0.5	−0.6	17	−0.3	+0.4	17	−1.7	+0.6
17	−1.3	−0.1	None regenerated at D under ligature. Five (those italicized) regenerated at P.			17	−0.3	−0.2	17	−1.2	0.0
17	−0.9	+0.2				None regenerated			All regenerated at D.		
All regenerated at D.											

Hours indicates number of hours since the stem was cut away from its hydranth. Measurements are in millivolts and were made between a point close to the distal end and a point in the middle (D-M). (P-M) indicates measurements made between a point close to the proximal end and a point in the middle.

reaching this stage. In this experiment and in all other experiments a relatively high negative value was found in regenerating regions in the period just before and during early primordium formation. Regions which were not going to regenerate developed lower negative potentials or positive ones.

In other experiments it was observed that regenerating ends became less negative or became positive during the late striate stage or when the base of the new hydranth was taking shape during what is called the pinched stage. This decrease in negativity with change to a positive charge had been observed by Barth (1934b).

A summary of typical potential differences between ends and middle are given for 3 stages in Figure 1. There is always high negativity prior to and during early regeneration at the distal end with a change of sign to a low positive reading for most by the time all levels of the primordium are delineated. The hours given for these stages are averages. Speed of regeneration varies considerably and striations first appeared as early as 17 hours and as late as 30 hours. It had already been observed by Barth (1934b) that when long stems were cut in thirds potential differences and speed of regeneration were greatest in distal thirds, less in middle thirds, still less in proximal thirds and the polarity was sometimes reversed in proximal thirds.

The figures given in Figure 1 are typical for rapidly regenerating stems. The figure given for the distal end of a typical 18 hour stem is −1.5 mv. The range for such preprimordium stems which subsequently completed regeneration was −0.9 to −1.7 mv. The non-regenerating proximal ends were less negative ranging from −0.8 mv to +0.6 mv. By the time striations had appeared at the distal end the proximal non-regenerating ends ranged from −0.4 to +0.4 mv. The distal ends where striations had appeared remained highly negative during the early striate stage.

Through the early stages of primordium formation up to the early striate stage high negativity in the range from −0.8 to −1.7 mv was found in regenerating

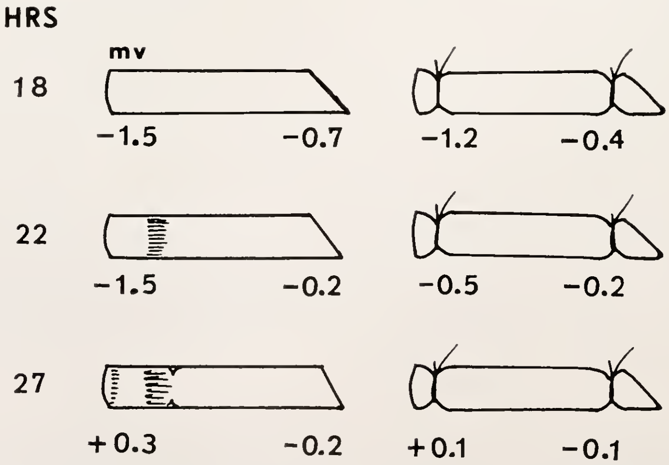


FIGURE 1. Potential differences in millivolts between ends and middle are compared in ligated and unligated stems at three different stages. The proximal ends are marked by an oblique cut.

ends. The dominated proximal ends showed values between −0.4 and +0.8 mv during the primordium stages.

In the cases covered in Table I and Figure 1 there was complete dominance of distal over proximal. When 10–15 mm pieces of stem were removed from colonies kept in the laboratory for several days or from colonies approaching dormancy in the harbor as the temperature approached 21° C, dominance was not complete. As a rule both ends transformed to hydranths. In these cases both ends became negative and remained so until the striate stage. The potential differences were lower in these more slowly regenerating stems showing decreased dominance. The range was from −0.5 to −1.0 mv. Other stems failed to regenerate. The highest negative value observed in them was −0.6 mv and the values ranged to +0.4 mv.

Ligatures at the ends of stems have an appreciable effect on potential differences measured along stems. They also prevent regeneration at the ligated ends. When stems were ligated at the distal end no regenerates appeared there. Five of eight stems released from the dominance of the distal end produced regenerates at the proximal end. At 12 hours three distal ends, even under their ligatures, had negative values of −0.9 to −1.4. These are in the range associated with incipient regeneration. Ligated distal ends failed to maintain high negativity and became less negative or became positive in the next few hours (Table I). At the same time some higher negative values were found in the no longer dominated proximal regions. The 3 proximal regions which failed to regenerate had negative values of 0.3, 0.6 and 0.8 mv. The one which had been −0.7 mv at 12 hours regenerated very slowly. Regeneration in the five stems which finally regenerated at the proximal end required 20–30 hrs longer than for regeneration at open distal ends. Polarity was reversed in these stems but required a long time.

The high negativity at the distal ligated ends at 12 hours was in the range found in incipient regenerators. It was already known that ligatures do not prevent the early pre-primordial stages of regeneration. When stems were ligated at both ends for a part of the pre-primordial period and their distal ligatures removed, they regenerated as rapidly as sister stems which had not been ligated (Rose and Rose, 1941).

Even in the stems ligated at both ends (Table I) two at 12 hours had potential differences normally correlated with incipient regeneration. After 12 hours the values decreased below the level found in rapidly regenerating stems.

The potential differences observed between ends and middle were very much alike in stems with both ends open and in stems with the proximal end ligated. In both cases all distal ends regenerated and all proximal ends failed to regenerate (Table I).

The above results demonstrate that high negativity is associated with regeneration. The major question was whether the basic reason for failure to regenerate was failure to maintain the relatively strong electrical polarity. Since it was already known that an applied field could cause a reversal in morphogenetic polarity in *Tubularia* (Barth, 1934a; Levin, 1961), it seemed possible that ligated stems with too low potential differences might be aided to regenerate by giving them a field to replace that which they were losing while ligated.

There were 7 experiments in which some ligated stems subjected to an electric field regenerated while ligated. Half faced the positive pole and half faced the negative pole. Seventeen of 79 with their distal ends facing the negative pole regenerated at their distal ends. None of 80 doubly ligated stems regenerated at their distal ends when they faced the positive pole. Four of these did regenerate at their former proximal ends which had faced the negative pole. In the 17 cases the applied field had acted in the same direction as the original inherent field. In the four cases in which regeneration had occurred at the proximal end the applied field had overcome the already weakened inherent field. Potential differences were measured along some of the ligated stems. The measurements were made in the period from 24 to 50 hours after the stems had been ligated. This was in the period before there was visible regeneration in most cases and when it was first observable in a few cases. Regeneration was considerably slower in ligated stems. Primordia did not appear until 48 to 60 hours.

It can be seen from Table II that 6 of the stems regenerated in which the distal end had faced the negative end of the field. Potential differences between distal ends and middle varied from −0.9 to −1.5 mv. Of the eleven measured stems treated in the same way, but which did not regenerate, the potential differences had been lower, ranging from −0.9 to +0.3 mv.

There were three of the measured stems which had faced in the other direction with their distal ends toward the positive pole which developed high negative potentials. These high readings were at the proximal ends toward the negative part of the applied field. Within 5 to 10 hours after the readings regenerates

Table II

The relationship between regenerative ability and negativity in stems ligated at both ends and subjected to a direct current field

Distal end faced negative pole				Distal end faced positive pole			
Regenerated at D(−)		Failed to regenerate		Regenerated at P(−)		Failed to regenerate	
D-M	P-M	D-M	P-M	D-M	P-M	D-M	P-M
−1.3	−0.9	−0.3	+0.3	+0.2	−1.6	−0.3	−0.3
−0.9	−0.5	−0.5	0.0	−0.1	−1.4	−0.1	−0.1
−0.9	+0.2	−0.4	+0.2	+1.2	−1.2	+0.5	+0.1
−1.1	+0.5	−0.5	−0.1			0.0	0.0
−1.0	+0.1	−0.6	0.0			−0.6	+0.9
−1.5	0.0	−0.8	+0.1			−0.7	+0.3
		+0.3	+0.4			−0.5	−0.1
		+0.2	+0.2			−0.2	−0.1
		−0.9	−0.1			−0.2	0.0
		−0.3	+0.3			−0.2	−0.3
		−0.7	+0.6			+0.5	+0.3
						+0.3	−0.4
						−0.7	−0.3
						−0.3	−1.1
						−0.5	−0.3
						−0.2	−0.7
						−0.8	−0.1

Readings in millivolts.

appeared at the proximal ends. This indicated that the applied field would not only maintain a field in the proper direction as in the former cases but that in these three cases the bioelectric field had been reversed. The regeneration was preceded by high negativity, 1.2 to 1.6 mv at the proximal ends.

Those doubly ligated stems which had faced the negative pole and did not regenerate had had a different range of potential differences, from −0.9 to +0.3 mv at their distal ends. The doubly ligated stems whose distal ends faced the positive pole ranged from −0.8 to +0.5 mv at the distal end and −0.7 to +0.9 mv at the proximal end.

The measuremetns were made in the period from a few minutes to 5 hours after the ligated stems had been removed from the imposed field. This means that in the successful regenerates the imposed field had caused a change in the bioelectric field which remained after the stems were removed from the imposed field.

In addition to the seven experiments in which some of the stems regenerated after being in or while still in the imposed field there were four other experiments in which no stems regenerated. Potential differences were measured in 32 of these stems. The highest negative value was −0.7 mv.

In all of the experiments a reading of −1.0 to −1.7 mv had predicted regeneration. A positive value or a negative value less than 0.7 mv had meant that no visible regeneration would follow. Values of −0.7 to −0.9 had no predictive value. Some regenerated and others failed. Potential differences are not steady and a stem with a reading of −0.7 to −0.9 mv could possibly have been slightly more or less negative than that most of the time.

In all of the experiments in which doubly ligated stems regenerated the stems had not been placed in the field immediately after ligation. After ligation they were held from one to 12 hours in a solution containing 10 mg of chloramphenicol per 100 ml of pasteurized sea water. The temperature was 15–16° C and the stems were not agitated. In general the longer, up to 12 hours, that the ligated stems were kept in this solution the greater was the effect of the subsequent treatment in the field.

A few stems were used from which small side branches had been removed flush with the main cylinder. Five such stems were ligated at both ends and the potential differences along the stem were measured shortly before a primordium could be expected to appear. The most negative region was the locus around the small opening in the perisarc. Just as Zwilling (1939) had learned after he had cut small openings in the perisarc of ligated stems, regenerates appeared in which all tentacles were aligned with their distal ends facing the small opening.

Discussion and Conclusions

In *Tubularia* any part of a stem can become a hydranth provided there is sufficient material. If there is too little to form a whole hydranth and just a part forms it is always a distal part. A small piece of totipotent tissue never forms anything but distal structures unless it lies behind a distal region. Then it forms the most distal structures not forming distal to it. The most distal region is the region from which organization spreads apparently by a series of repressions (Rose, 1957, 1967b, 1970).

The results of this work indicate that the most negative locus becomes the distal center of organization. Openings through the perisarc either at ends or along stems become negative centers. There is also an inherent electrical gradient with distal regions being more negative. If a distal region is ligated, a proximal end near the opening becomes the most negative. Imposed fields can also induce negative regions. Whatever the cause of the high negativity, the negative locus becomes a center of organization.

High negativity is necessary for regeneration. In ligated stems the negativity decreases to a level not found in regenerating stems. If the proper level of negativity is maintained in an artificial field, regeneration can proceed even under ligatures.

In addition the sign of an applied field can determine the sign of a bioelectric field and the sign of regeneration.

The measurements in this work were made at the external surface of the perisarc because of the relative ease with which this can be done. Lund (1925) had shown that the sign of the internal potentials is the reverse of that recorded at the surface. This means that internally where the repressors would be expected to move, the distalmost region is positive during the period when the repressors act. The internal bioelectric field would cause them to move from the positive distal region to the more negative proximal region.

It now seems clear that the bioelectric field is necessary for regeneration and not just a symptom of differences in chemical activity.

Summary

Electrical potential differences were measured in isolated stems of *Tubularia* during transformation of parts of the stems to hydranths. The transforming regions during the early stages of primordium formation were electronegative at the surface to adjacent non-transforming regions.

Potential differences lower than those measured in regenerating stems were obtained from non-regenerating ligated stems.

Ligated stems which were failing to regenerate because their potential differences were too low were given potential differences as great as those found in regenerating stems. When they were placed in an artificial field of appropriate strength, the ligated stems which regained the proper potential differences regenerated.

LITERATURE CITED

Barth, L. G., 1934a. The effect of constant electric current on the regeneration of certain hydroids. *Physiol. Zool.*, **7**: 340–364.

Barth, L. G., 1934b. The direction and magnitude of potential differences in certain hydroids. *Physiol. Zool.*, **7**: 365–399.

Child, C. M., and L. H. Hyman, 1926. Studies on the axial gradients in *Corymorpha palma*. *Biologia Generalis*, **2**: 355.

Hyman, L. H., 1920. The axial gradients in Hydrozoa. III. Experiments on the gradient of *Tubularia*. *Biol. Bull.*, **38**: 353.

Hyman, L. H., and A. W. Bellamy, 1922. Studies on the correlation between metabolic gradients, electrical gradients and galvanotaxis. *Biol. Bull.*, **43**: 313.

Levin, S., 1961. Anodal inhibition of the regeneration of *Tubularia crocea* within an electric current. *Biol. Bull.*, **121**: 305.

LUND, E. J., 1921. Experimental control of organic polarity by the electric current. I. Effects of electric current on regenerating internodes of *Obelia commisuralis*. *J. Exp. Zool.*, **34**: 471–493.

LUND, E. J., 1925. Experimental control of organic polarity by the electric current. V. The nature of the control of organic polarity by the electric current. *J. Exp. Zool.*, **41**: 155–190.

MARSH, G., AND H. W. BEAMS, 1952. Electrical control of morphogenesis in regenerating *Dugesia tigrina*. I. Relation of axial polarity to field strength. *J. Cell. Comp. Physiol.*, **39**: 191–213.

MATHEWS, A. P., 1903. Electrical polarity in the hydroids. *Amer. J. Physiol.*, **8**: 295–299.

MILLER, R. L., 1969. Identification of Woods Hole species of *Tubularia*. *Biol. Bull.*, **137**: 409.

ROSE, S. M., 1957. Polarized inhibitory effects during regeneration in *Tubularia*. *J. Morphol.*, **100**: 187–205.

ROSE, S. M., 1963. Polarized control of regional structure in *Tubularia*. *Dev. Biol.*, **7**: 488–501.

ROSE, S. M., 1966. Polarized inhibitory control of regional differentiation during regeneration in *Tubularia*. II. Separation of active materials by electrophoresis. *Growth*, **30**: 429–447.

ROSE, S. M., 1967a. Polarized inhibitory control of regional differentiation during regeneration in *Tubularia*. III. The effects of grafts across sea water-agar bridges in electric fields. *Growth*, **31**: 149–164.

ROSE, S. M., 1967b. The aging of the system for the transmission of information controlling differentiation. *J. Gerontology*, **22** (II): 28–41.

ROSE, S. M., 1970. Differentiation during regeneration caused by migration of repressors in bioelectric fields. *Amer. Zool.*, in press.

ROSE, S. M., AND F. C. ROSE, 1941. The role of a cut surface in *Tubularia* regeneration. *Physiol. Zool.*, **14**: 328–343.

TARTAR, V., 1964. Morphogenesis in homopolar tandem grafted *Stentor coeruleus*. *J. Exp. Zool.*, **156**: 243–252.

ZWILLING, E., 1939. The effect of the removal of perisarc on regeneration in *Tubularia crocea*. *Biol. Bull.*, **76**: 90–103.

Reference: *Biol. Bull.,* **138**: 354–378. (June, 1970).

THE BLOOD CELLS AND TUNIC OF THE ASCIDIAN *HALOCYNTHIA AURANTIUM* (PALLAS). I. HEMATOLOGY, TUNIC MORPHOLOGY, AND PARTITION OF CELLS BETWEEN BLOOD AND TUNIC[1]

MICHAEL J. SMITH[2]

Department of Zoology, University of British Columbia, Vancouver 8, Canada

The blood cells and tunics of a number of ascidians have been described (Seeliger and Hartmeyer, 1911; Hecht, 1918; St. Hilaire, 1931; George, 1939; Endean, 1955a, 1955b, 1960, 1961; Andrew, 1962; Freeman, 1964). There generally appears to be three categories of blood cells present: vacuolar, amoebocytic, and a lymphocytic stem type. High levels of transition metals have been reported in the blood cells of tunicates (Henze, 1911; Webb, 1939, and 1956; Bertrand, 1950; Endean, 1955a, 1955b, 1960, and 1961; Levine, 1962). Several functions have been ascribed to the blood cells of ascidians: tunic secretion (Ries, 1937; Endean, 1955b, and 1961; Kalk, 1963), phagocytosis, defense response to foreign bodies (Anderson, 1969) and trephocytosis (Liebman, 1947). It has been recognized for some time that the blood cells move into, and take up residence in, tunic material (Seeliger and Hartmeyer, 1911; Hecht, 1918; Endeon, 1955a, 1955b, 1960, and 1961). There is some disagreement concerning the source of the tunic, whether it is a blood cell secretion (Endean, 1955b, and 1961) or an epidermal secretion (Deck, Hay, and Revel, 1966). The morphology of the tunic of various ascidians is diverse (St. Hilaire, 1931) and this has complicated the problem of understanding the origin of tunic and blood cell function therein. There has been little quantitative consideration or statistical analysis concerning blood cell distribution and function.

The solitary ascidian, *Halocynthia aurantium* (Pallas), is commonly called the sea peach because of its coloration and general shape. It is considered to be the Pacific counterpart of the Atlantic species (*Halocynthia pyriformis* (Rathke) (Van Name, 1945). In Howe Sound, British Columbia, *H. aurantium* is seldom found at a depth of less than 10 meters and the animals are found in their greatest numbers between 10 and 20 meters; the greatest depth at which *H. aurantium* was encountered by the author was approximately 25 meters. At these depths the animal is not exposed by tidal fluctuations and it is below the brackish surface layers that occur in Howe Sound during Spring and Summer freshwater runoff. *H. aurantium* appears to prefer attachment to vertical rock faces exposed to the currents. *H. aurantium* is found in large populations which contain extensive size ranges of individuals, but these populations are distributed erratically through Howe Sound. In this species, the tunic is clean and free of detritus and settling or boring organisms. The gastropod mollusc, *Fusitriton oregonensis,* has been observed to prey upon *H. aurantium* both in the field and in the lab, but this is a rare occurrence and it is believed that *H. aurantium* is relatively free of predation (Smith, unpubl. obs.). *H. aurantium* accumulates iron with the greatest concentration per gram dry weight found in the blood cells and with the second highest concentration found in the tunic (Smith, 1969). This study was undertaken to determine the morphology of the blood cells and tunic of this hitherto uninvestigated ascidian. Statistical analyses of blood cell distributions, their partition in the tunic, and their reaction to experimental manipulation enable the formulation of tentative hypotheses concerning the relationships of certain blood cell types, the origin of the tunic, and the function of particular cell types in the tunic.

---

[1] This work was supported by grants from the National Science Foundation (#GB 4358) and the National Research Council of Canada (#T88) to Dr. P. A. Dehnel of the University of British Columbia.

[2] Present address, Department of Zoology, University of Nebraska, Lincoln, Nebraska. This work represents a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. at the Department of Zoology, University of British Columbia.

Materials and Methods

The ascidian, *Halocynthia aurantium* (Pallas), was identified by the author based on the work of Van Name (1945). The ascidians were collected in the late summer and early fall of 1966 by scuba diving at 10 to 20 meters from the following locations in Howe Sound, British Columbia: Whytecliff Park, Copper Cove, Horseshoe Bay, Britania Beach, and Sunset Beach. Ascidians were held in the lab in constantly aerated sea water at 10° C. or 14° C for at least five days prior to experimentation. The ascidians were squeezed gently until sea water ceased to flow from the siphons, blotted dry, and weighed. Wet weights of whole animals were determined on a triple beam balance to 0.01 g. Dry weights to 0.1 mg were determined on a Mettler balance after the animals were dried for 48 hours at 110° C.

The following statistical methods were employed; analysis of variance, Keul's sequential test for mean differences, linear regression calculation and tests of the significance of calculated regressions, correlation coefficients (r), Student t-test, and t-test for mean differences in paired observations (Snedecor, 1956; Steel and Torrie, 1960; Li, 1964). Since per cent data tend to be binomial, all such data, which were to be used for tests of significance, were transformed to the angle equal to the arc sine square root of per cent (Li, 1964). F-ratios were considered significant if the probability that the event was a random occurrence was equal to or less than 0.05.

A small longitudinal incision was made in the subendostylar sinus area of the ascidian, a drop or two of blood was collected and mixed with a drop of 5% formalin in sea water on a clean microscope slide. The preparation was allowed to stand for one minute, smeared across the slide, and stained with Leishman's stain in methanol. After staining, the smear was very rapidly dehydrated through two changes of 100% tertiary butanol, one change of 50% t-butanol 50% xylene, cleared through two changes of xylene, and mounted in "Permount." Slides prepared in this manner display good stain differentiation, but the staining quality deteriorates within several weeks.

For fixation of tissues or whole animals, buffered formalin with cetyl pyridinium bromide in distilled water (Culling, 1963) was used. Other fixatives employed were 5% formalin in sea water, Bouin's, Baker's, and Carnoy's (Culling, 1963). Whole animals or tissues were fixed for 4 hours in Carnoy's and 24 hours in all other fixatives. After fixation, tissues were washed, dehydrated in graded ethanols, cleared in benzene, and embedded in Paraplast (M.P. 55.6° C.). Trimmed blocks were cut at 8 microns on a Spencer microtome. Frozen sections were cut from material, which had been fixed for 12 hours in cetyl pyridinium formalin, at 10 to 16 microns on an International cryostat.

Although several hematoxylin stains were attempted, the chrome hematoxylin with phloxine method of Gomori (1941) was found to be most satisfactory. An alternative stain for tunic material was aldehyde fuchsin with light green (Pearse, 1961). For the histochemical determination of iron, Perl's and Turnbull's methods for ferric iron and the dinitroresorcinol method of Humphrey were employed (Pearse, 1961). Some sections to be stained for iron were treated with 30% hydrogen peroxide and 5% oxalic acid to unmask protein bound iron (Dales, 1965). Osmium fixed blood cells were prepared by exposing fresh blood smears to osmium tetroxide vapors in a sealed coplin jar for one hour after which the smears were dehydrated, cleared, and mounted.

Differential cell counts were made from duplicate smears of blood from wet weighed animals. 100 cells per slide or 200 cells per ascidian were counted. Mean differential distributions of blood cells were obtained for 80 animals over a weight range from less than 1 to 80 grams. The group of 80 animals was broken into four sub-groups of twenty gram weight range each containing 20 animals. Per cent distribution values were transformed by the arc sine method, and the distribution of particular cell types was tested by the analysis of variance for differences as a function of weight group. If a significant F ratio was obtained for any cell type, the weight group means for that cell type were tested for mean differences by Keul's method.

Hematocrits were measured in Kolmer centrifuge tubes after 10 minutes centrifugation at 3000 rpm on an Orbit centrifuge. Total number of cells per cubic millimeter of blood was determined for 35 animals as a function of wet weight group by duplicate hemacytometer counts. Blood was diluted with 5% formalin in sea water which was 0.1% in eosin. Plasma pH was determined directly with a Beckman research model pH meter at room temperature.

Tunic was injured by extirpating a 5 by 10 mm section of tunic without damaging the epidermis. Samples with damaged epidermis were not used for cell counts. At 0, 1, 3, 5, 10, and 15 days after injury, six ascidians were fixed and the injured area was removed for sectioning. Sections of tunic and epidermis were cut at right angles to the long axis of the ascidian. All the cells in the tunic, from the epidermis to the exterior were counted as a function of their position relative to the epidermis in terms of numbers of cells per ocular field from the epidermis. The distance from epidermis to exterior was measured with an ocular micrometer, the field diameter was known, and the width of section (8 microns) was known. Consequently, cell counts could be expressed as number of cells per mm^3. of tunic and were comparable with blood concentration. Stolons, finger-like villi that form the hold-fast, were sectioned at right angles to their long axis. Cell counts in the tunic of stolons grown in the laboratory were made at the half distance from the growing tip of the stolon to the body wall of the animal. The morphology of body wall tunic and the tunic of natural and laboratory grown stolon was examined in serial cross section and representative longitudinal sections. All tunic material used for comparisons of cell concentration was from animals weighing 20 to 40 grams and was compared with blood values of that weight range.

A suspension of Aqua-dag colloidal carbon, 5% solids in sea water, was injected into the tunic of the right lateral area of a group of 20 to 40 gram ascidians. At 0, 3, 6, 12, 24, and 48 hours subsequent to injection, blood was drawn from the subendostylar sinus of six animals per time period and fresh and Leishman's stained smears were prepared. For each animal two smears were prepared and approximately 200 cells per animal were counted. The per cent and type of cell with carbon particles was determined. Per cent data were transformed by the arc sine method and analysis of variance and mean comparisons as a function of time after injection were calculated.

RESULTS

I. Wet vs *dry weight*

There is a linear relationship between wet and dry weights of *H. aurantium*. The calculated correlation coefficient (r) is +0.997 (n = 29). Regression analysis reveals a slope value of 0.065. The probability that the calculated slope is due to random variability is less than 0.001.

II. Blood cell morphology

A. Mature morula cells (Fig. 1A) comprise 2.9% of the blood cells based on a sample of 80 animals. They are between 9 and 10 microns in diameter with an anucleolar nucleus. The mature morula cell is filled with 1 to 2 micron vacuoles. The number of these vacuoles varies, but is seldom less than twenty. Under bright field illumination this cell is highly refractive and light green in color. The contents of the vacuoles are homogeneous under both phase and bright field illumination and acidophilic to Leishman's stain. Osmium vapors cause a light brown to gray coloration of the cell. The mature morula cell is distinguished from other vacuolar cells by the small size and large number of acidophilic vacuoles, all of which are smaller in diameter than the nucleus.

B. Immature morula cells (Fig. 1B), which are approximately 9 microns in diameter, comprise 28.1% of the blood cells. The cell is filled with 5 to 15 vacuoles which are each approximately equal to the nucleus in diameter (3 microns). Staining reactions are similar to the mature morula cells: vacuoles are acidophilic, green in live cells, and turn gray after exposure to osmium vapors. The number of vacuoles is lower than in mature morula cells and the vacuoles are consistently larger. Transition stages between the immature and mature morula cells are considered immature morula cells if any of the vacuoles present are equal to or larger than the nucleus in diameter and are five or more in number.

C. Compartment cells (Fig. 1C) make up 1.0% of the blood cells, are about 8 microns in diameter, and have 2 to 4 vacuoles which are larger than the nucleus. The contents of the vacuoles are homogenous and staining reactions are similar to

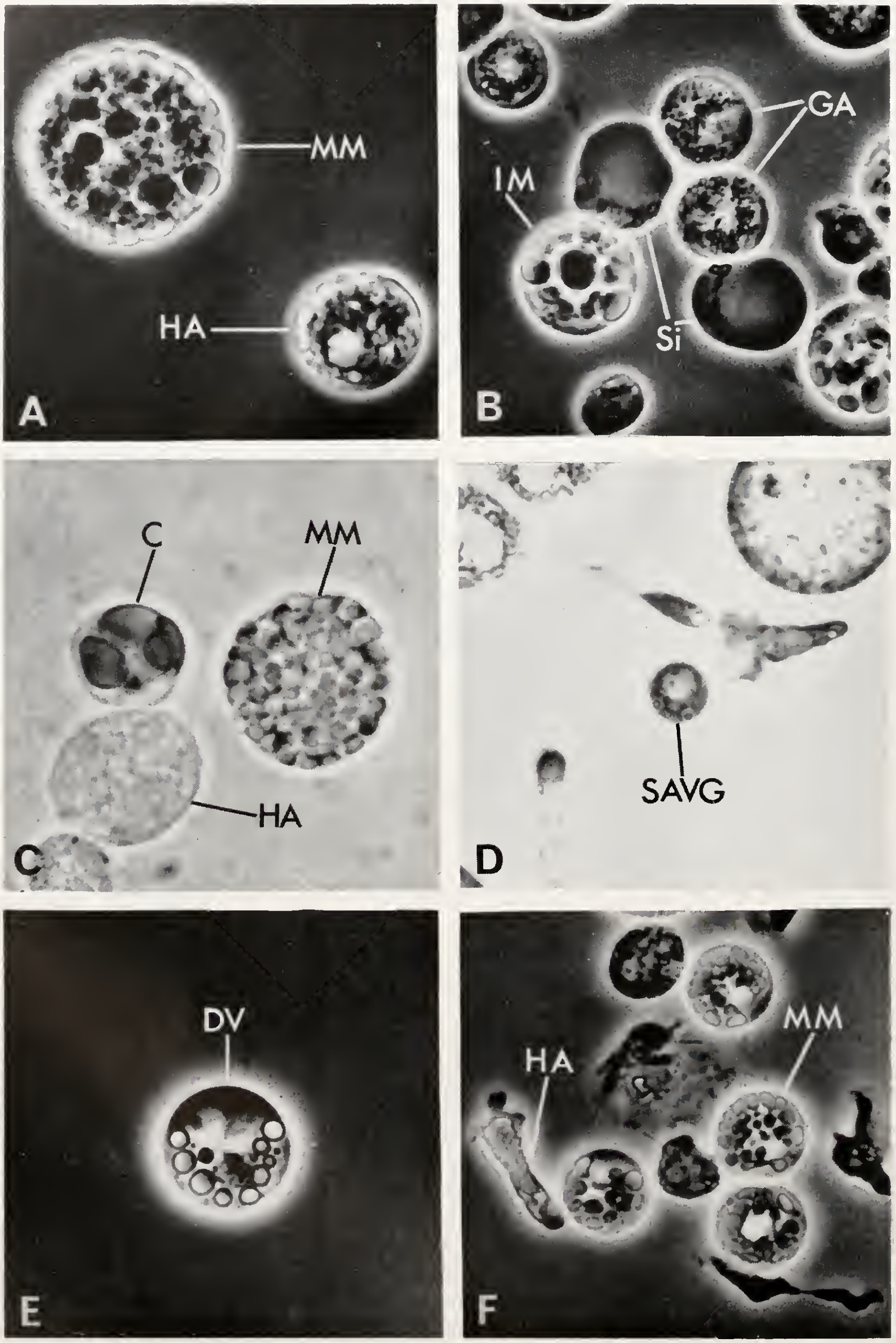


FIGURE 1. The blood cells of *H. aurantium*. Plates A (3000 ×), B (3000 ×), D 2000 ×), E (2000 ×), and F (1250 ×) are photographs of live cells taken through phase optics. Plate C (2000 ×) is a photograph of Leishman stained smear taken through bright field optics. The following notation is used: MM, mature morula cell; IM, immature morula cell; GA, granular amoebocyte; Si, signet ring cell; HA, hyaline amoebocyte; C, compartment cell; SAVG, stem cell with acidophilic vacuoles or granules; and DV, dispersed vesicular cell.

the immature morula cell. No nucleoli were observed in this cell type. These cells are distinguished from the immature morula cell by the larger size and lower number of vacuoles.

D. Signet ring cells (Fig. 1B) have one vacuole which fills the cytoplasm, pressing the nucleus to one side. They comprise 1.7% of the cells in circulation and are on the order of 6 microns in diameter. A cell is considered a signet ring when the vacuole is larger than the nucleus and when the vacuole does not contain granular inclusions. The vacuole is acidophilic or, more rarely, neutrophilic. The nucleus may occasionally contain a small nucleolus.

E. *Hyaline amoebocytes* (Fig. 1A, C, F) comprise 38.5% of the blood cells and range from 6 to 12 microns in diameter. The nucleus occasionally has a nucleolus. The cytoplasm is homogeneously neutrophilic to Leishman's stain. The cell may contain one or more vacuoles which enclose a small granular inclusion that is subject to Brownian motion in live cells. The cells may contain a large yellow grain. Under phase illumination, the cytoplasm appears reticulated or clumped. These cells are extremely amoebocytic and assume a variety of bizarre forms when viewed in live smears.

F. *Granular amoebocytes* (Fig. 1B) make up 20.5% of the cells in circulation. They have a discrete size range of 6 to 8 microns and are packed with distinct granules approximately 0.2 microns in diameter. The granules have some refractive properties when viewed under bright field illummation. Leishman's stained smears and phase contrast studies show that the granules do not fill the cytoplasm but leave a peripheral hyaline area. The granules are neutrophilic. Under phase illumination, the granules are seen to be associated with a reticular network.

G. *Stem cells* are 5 to 6 microns in diameter and make up 4.8% of the blood cells. The nucleus, which almost fills the cytoplasm, contains one or two small nucleoli. The chromatin is dispersed and the cytoplasm stains slightly basophilic with Leishman's stain. Under bright field and phase illumination, the cytoplasm of live cells appears hyaline and the nucleus looks clear except for a dark nucleolus.

H. *Stem cells* with acidophilic vacuoles or granules (Fig. 1D) are essentially similar to the stem cell except that they may be larger (5 to 7 microns), have a smaller nucleus with nucleolus, and have vacuoles and/or granules which are acidophilic to Leishman's stain. Vacuoles, when present, are always smaller than the nucleus.

I. *Giant stem cells* are rarely observed in the blood, and comprise only 0.2% of the cells. They are quite large (9 to 10 microns in diameter) with a large nucleus (5 microns) and a large nucleolus. The staining reactions of the giant stem cells are essentially similar to the stem type. Live giant stem cells are actively amoeboid and their cytoplasm is generally homogeneous, although occasionally it displays a fine granulation.

J. *Dispersed vesicular cells* (Fig. 1E) are of a uniform size (8 microns) and the cytoplasm contains 8 to 12 discrete vesicles of approximately 1 micron in diameter which are regularly dispersed throughout the cytoplasm. The vesicles are difficult to observe in live cells under bright field illumination, but under phase contrast they appear light against a dark background. With Leishman's stain, the cytoplasm is faintly basophilic and the vesicles faintly acidophilic. With

Gomori's chrome hematoxylin, the vesicles stain an intense blue-black. If the oxidation step of the procedure is omitted, the vesicles stain with phloxin. The nucleus often has a nucleolus.

None of the blood cells in *H. aurantium* stain for the presence of iron unless the sections are treated with hydrogen peroxide and oxalic acid. Subsequent to such treatment, the signet ring, the compartment, immature morula, and mature morula cells stain for iron. The staining intensity varies both within cell types and between cell types. This is probably due to a loss of the metal as it is unmasked by the action of the reagents.

III. Hematology

The mean number of cells per mm^3 of blood from *H. aurantium* is 17.240 ($n = 35$, S.E. = 1690). Analysis of variance for mean number of cells per mm^3 as a function of 20 gram wet weight group displays an insignificant F ratio. The mean hematocrit is 0.38% ($n = 15$, S.E. = 0.06%). The mean corpuscular volume is 220 cubic microns. Mean plasma pH is 7.31 ($n = 6$) with a range from 7.20 to 7.41.

The mean differential distribution of blood cell types, derived from the 80 animal sample, is given with the description of the cell types (see above). The analyses of variance of per cent distribution of particular cell types as a function of 20 gram wet weight group gives significant F ratios in the following categories of cells: mature morula, stem, stem with acidophilic vacuoles or granules, dispersed vesicular cells, and hyaline amoebocytes (Table I). The compartment cells, immature morula, and signet ring cells do not show significant F ratios as a function of weight group when considered as individual types. If these cells, however, are grouped together into a category of *vacuolar cells,* the analysis of variance gives a significant F ratio as a function of weight group (Table I). The stem, stem with acidophilic vacuoles or granules, dispersed vesicular cells, mature morula, and *vacuolar cells* decrease with increased weight of animal (Fig. 2). The hyaline amoebocytes increase with increased weight of animal (Fig. 3). The 20–40,

TABLE I

The relative concentration of blood cell types in the blood of H. aurantium *as the mean per cent of particular cell type for each twenty gram wet weight group. The F ratio and P level from analyses of variance of per cent distribution of particular cell types as a function of wet weight group is given*

Cell type	Mean per cent*				F	*P*
	0–20	20–40	40–60	60–80		
Mature morula	4.8	2.5	2.4	1.7	6.188	<0.005
Dispersed vesicular	2.6	1.9	1.2	0.9	3.969	<0.025
Vacuolar	35.2	31.7	27.0	27.4	2.789	<0.05
Stem	6.7	4.7	4.7	2.9	3.752	<0.025
S.A.V.G.	1.8	0.9	0.9	0.5	7.173	<0.005
Hyaline amoebocyte	27.7	37.3	45.1	45.1	13.286	<0.025

*Mean per cent of cell type in the indicated weight range (g).

40–60, and 60–80 gram weight groups have significantly higher numbers of hyaline amoebocytes than the 0–20 gram group. The levels of significance of difference of mean per cent, after transformation by the arc sine method, of stem cells, stem cells with acidophilic vacuoles or granules, dispersed vesicular cells, mature morula, and vacuolar cells are given in Table II.

IV. The Epidermis

The epidermis (Fig. 4B) is a columnar epithelium with basophilic cytoplasm. The nuclear chromatin is diffuse and a nucleolus is rarely observed. The cells measure approximately 6 by 8 microns and frontal sections show that they are

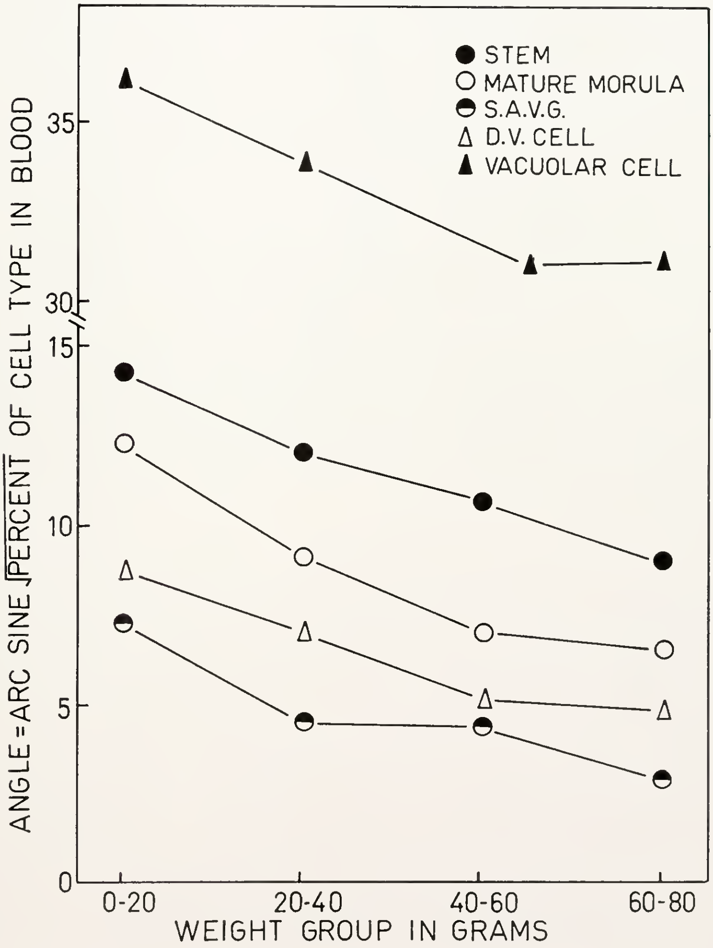


FIGURE 2. The per cent distribution of blood cell types in blood as a function of 20 gram whole animal wet weight groups of *H. aurantium*. Each point is the mean value for twenty animals in that weight group. *Vacuolar* cells refers to a collective group including the immature morula, compartment, and signet ring cell types.

TABLE II

The significance of differences (Keul's method) of mean per cent of various blood cells as a function of 20 gram wet weight group in H. aurantium

Weight range	Cell type	PX Difference		
		−(60–80)	−(40–60)	−(20–40)
0–20 grams	Stem	0.01	0.01	0.01
	S.A.V.G.	0.01	0.01	0.01
	Vacuolar	0.01	0.01	0.01
	M.M.	0.01	0.01	0.01
	D.V.C.	0.01	0.01	0.01
20–40 grams	Stem	0.01	0.01	
	S.A.V.G.	0.01	N.S.	
	Vacuolar	0.01	0.01	
	M.M.	0.01	0.05	
	D.V.C.	0.01	0.01	
40–60 grams	Stem	0.01		
	S.A.V.G.	0.01		
	Vacuolar	N.S.		
	M.M	0.01		
	D.V.C.	N.S.		

polygonal; each cell is generally surrounded by six other cells. No pores, channels, or vessels extend through the epidermis of the body wall into the tunic. Fibers of tunic material protrude from the apical ends of the epidermal cells. The epidermis is attached to a basement membrane from which connective tissue sheets extend through the subepidermal blood space to the circular and longitudinal muscles of the body wall.

V. Tunic

The thickness of the tunic removed from fixed animals displays great variation, ranging from 0.60 mm for a 1.5 gram animal, to 1.20 mm for a 60 gram ascidian.

The greatest portion of the tunic is made up of a fibrous matrix (Fig. 4A) in which blood cells may be observed. The fibers, which extrude from the apical ends of the epidermal cells, coalesce just distal to the epidermis to form a fibrous layer or lamina. The matrix of tunic consists of a regular arrangement of these laminae, which form successive sheets of tunic material around the animal. The laminae are joined together by fibrous extensions between them. In larger animals, the laminae are grouped into bands; within a band, laminae are approximately of the same thickness, but the dimensions of laminae between bands differ. Cross sections of tunic from larger animals show bands of thick and thin laminae. There is a positive correlation between the number of laminae and wet weight of animal, $(r) = +0.95$ $(n = 28)$. Linear regression analysis of the number of laminae as a function of wet weight gives a slope of 1.67 and an intercept of 25.5. Confidence limits at the 0.95 level for the estimation of number of laminae from a given weight have been calculated (Fig. 5).

The external surface of the tunic is formed of papillar outgrowths of the matrix, upon which are situated cuticular spinous processes (Fig. 4C). Towards the periphery of the tunic the laminar structure is lost gradually and the fibers extend outwards forming a papilla. The mean height of papilla and cuticular spine is 271 microns (n = 11, S.E. = 28 microns). The spine is approximately 160 microns (n = 11, S.E. = 7 microns) in width at its base. The spine tapers from its base to an extremely fine point. The fibers of the matrix pass up

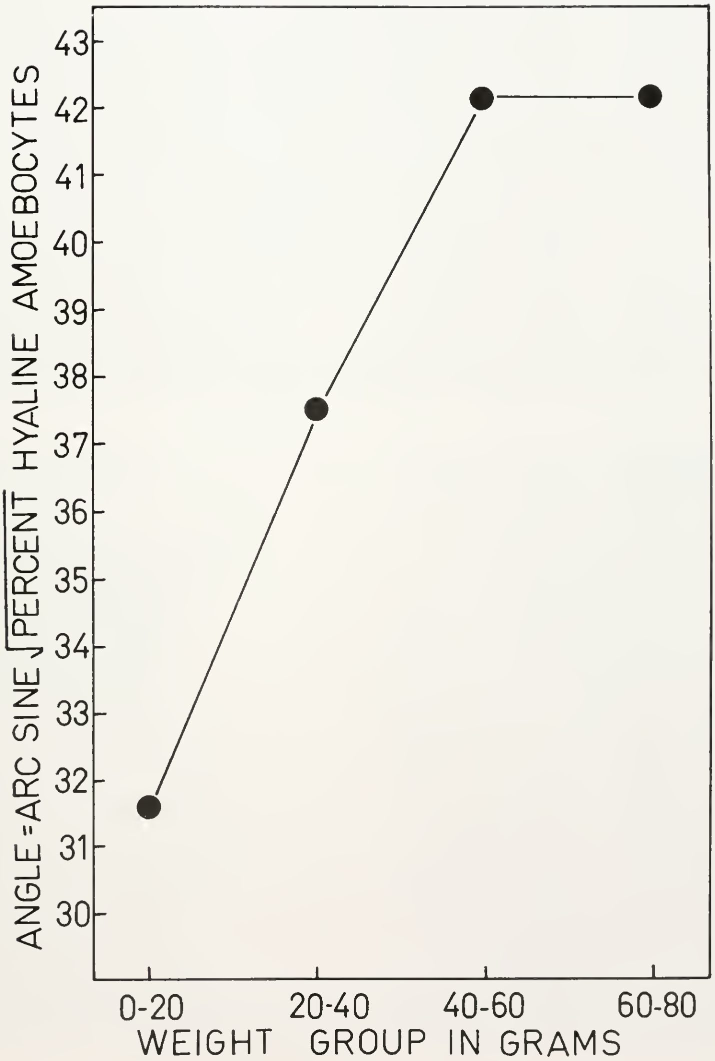


FIGURE 3. The per cent distribution of hyaline amoebocytes in the blood of *H. aurantium* as a function of 20 gram whole animal wet weight group. Each point is the mean value determined for 20 animals in that weight group.

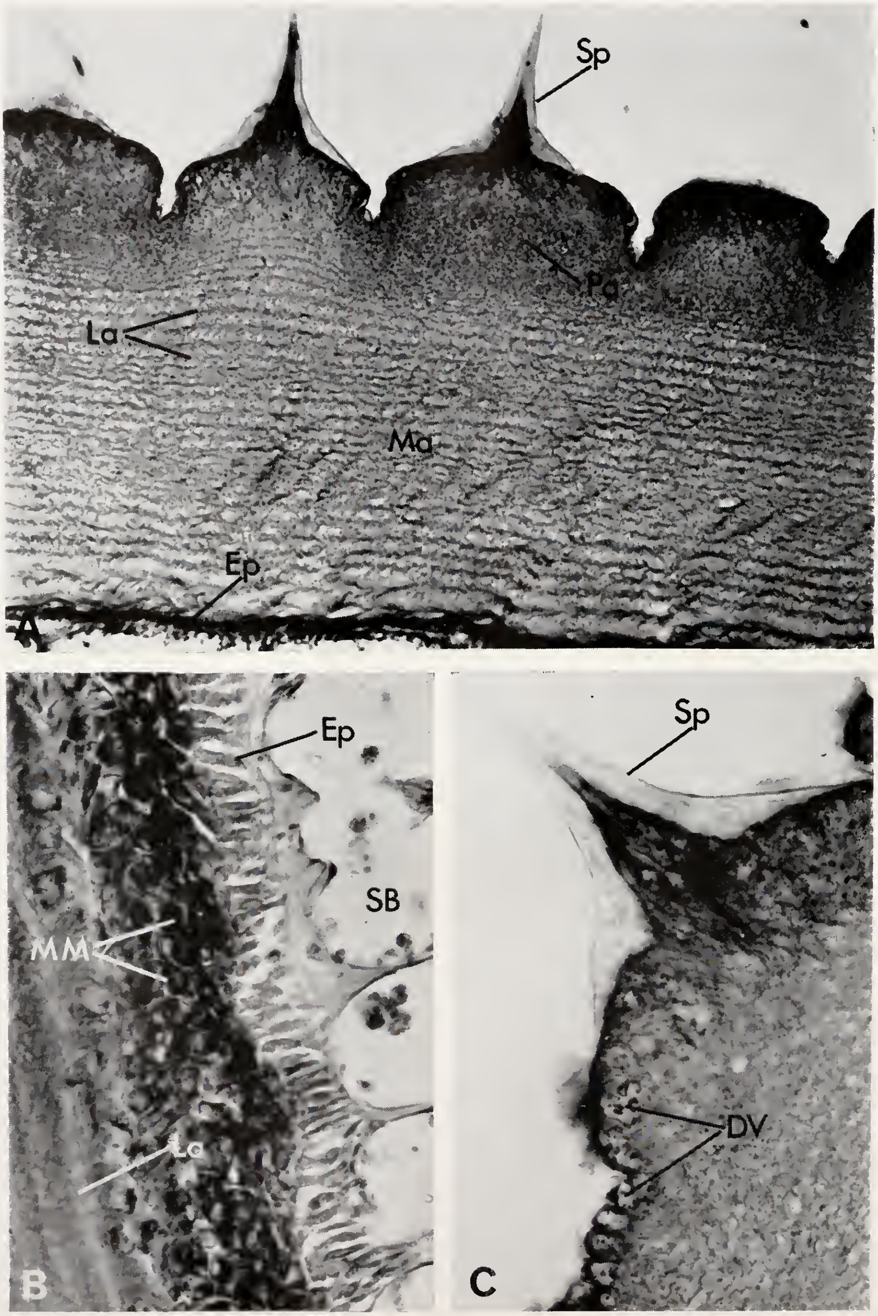


FIGURE 4. The tunic of *H. aurantium* stained with Gomori's hematoxylin and phloxine. Plate A (120 ×) is a cross section of body wall tunic. Plate B (650 ×) is a cross section at the level of the epidermis with an aggregation of mature morula cells just above the epidermis. Plate C (400 ×) is a cross section of a papilla bearing a tunic spine. The following notation is used: Sp, tunic spine; La, laminae; Ma, matrix; Ep, epidermis; SB, subepidermal blood space; MM, mature morula cell; and DV, dispersed vesicular cell.

the spine for some distance. The spine has a hollow core which passes all the way to the tip, and often contains blood cells. The material of the spine is homogeneous. Between the papillae, the surface of the matrix is overlaid with a thin layer of dense staining cuticle.

VI. The stolons

The hold-fast of *H. aurantium* consists of a number of stolons (Fig. 6A) which are finger-like outgrowths of the tunic, the underlying epidermis, and subepidermal blood spaces. The epidermis of stolons is more cuboidal than that of

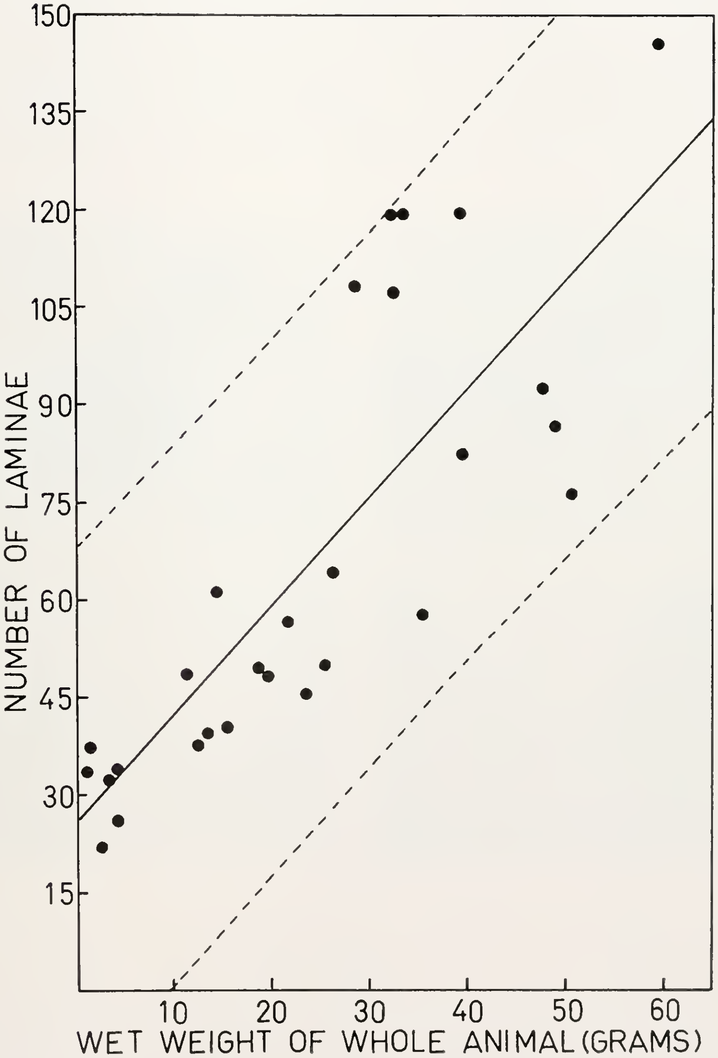


FIGURE 5. The number of laminae, or fibrous layers, in the tunic as a function of whole animal wet weight in grams. The solid line represents the calculated regression line. The dashed line represents the 95% confidence limits in the estimation of a y value for any given x value. The correlation coefficient is +0.95.

the body wall. The tunic matrix material of natural hold-fast stolon is irregular and tunic papillae and spines are absent although a dense staining cuticular surface is evident. In the laboratory, stolon material grows freely and rapidly, circumscribing the base of the ascidian. Over several months, these stolons will grow to lengths of approximately 1 mm and to diameters of 0.1 to 0.2 mm. The tunic matrix fibers of laboratory stolon show more regularity of structure than does the natural stolon matrix. At the distal tip of the laboratory grown stolon, the matrix material is quite thin and irregular (Fig. 6A). Proceeding from this tip along the stolon to the body of the animal there is an increase in the thickness of the matrix and an increase in the regularity of fibrous structure, until the matrix assumes a laminar appearance like that found in body wall tunic. The growing tip of laboratory stolon is covered with a thin cuticle, but near the body of the animal, papillae and cuticular spines are evident. There is a gradation of development of matrix, papillae, and cuticular spines from the distal tips of stolons to its juncture with the body wall.

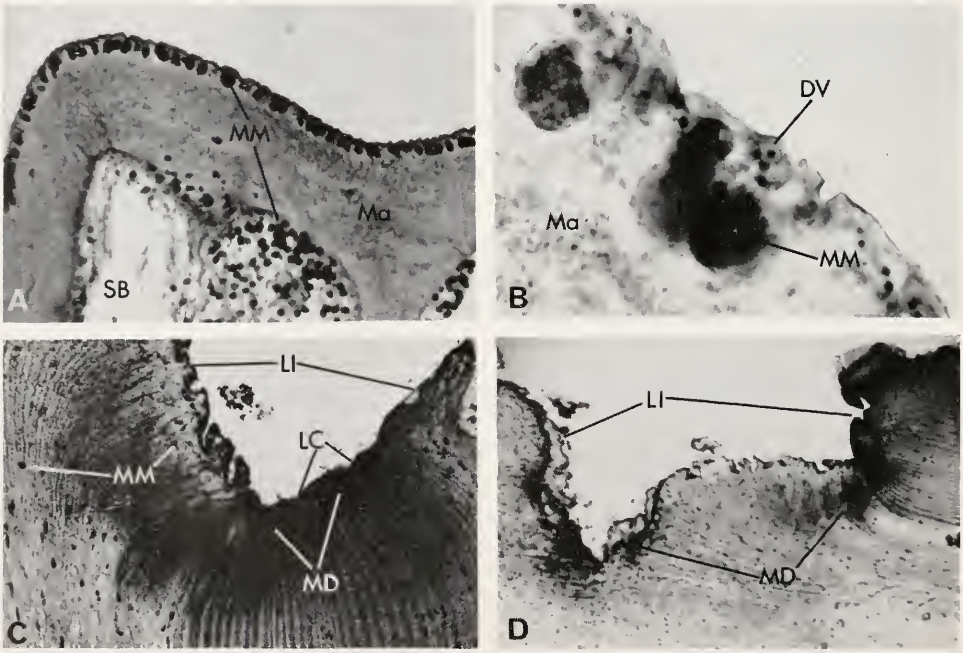


FIGURE 6. Laboratory grown stolon and injured body wall tunic of *H. aurantium* stained with Gomori's hematoxylin and phloxine. Plates A (200 ×) and B (1000 ×) are cross sections of stolon near the growing tip; note the aggregation of mature morula cells (MM) and dispersing vesicular cells (DV) peripherally and the lack of lamination of the tunic matrix (Ma). Plates C (100 ×) and D (50 ×) are cross sections of tunic five days after injury. In plate D the epidermis is to the bottom; note the dark staining mature morula cell degradation products (MD) throughout the wound area and the constriction of laminae (LC) at the sides of the wound. The following additional notation is used: SB, subepidermal blood space; LI, limits of original incision.

VII. Blood cells in tunic

Four blood cell types concentrate in tunic: mature morula cells, dispersed vesicular cells, granular amoebocytes, and hyaline amoebocytes. Comparisons of blood cell concentrations in tunic, stolon tunic, and blood demonstrate that all four cell types are present in tunic at significantly higher concentrations than in blood (Table III). In tunic, all four cell types are present in greater concentration in the stolon than the body wall (Table III). Within body wall tunic, the mature morula and dispersed vesicular cells show discrete positional relationships (Figs. 4B, 7). Mature morula cells are rare in the outer areas of tunic, but are highly concentrated in tunic adjacent to the epidermis. The dispersed vesicular cells are evident in small numbers throughout the tunic and are present in extremely high concentrations at the external periphery of the tunic, particularly in inter-papillary areas. The hyaline and granular amoebocytes do not have positional sites of concentration in the tunic.

Both the mature morula and dispersed vesicular cells undergo morphological changes in the tunic. The mature morula breaks down in the tunic, either by a dispersion of its vacuoles, as if the cell membrane had been disrupted, or by a coalescence of the smaller vacuoles into one or two large vacuoles which appear to be empty. Mature morula cells which have undergone these changes in the tunic are referred to as *tunic vesicular cells.* There is no analogous break down of mature morula cells in the blood. In the tunic, the vesicles of the dispersed vesicular cell are smaller and more difficult to stain, the cytoplasm loses its affinity for stain, and the nucleus appears pycnotic.

VII. Carbon phagocytosis

Hyaline amoebocytes are the only cells which have engulfed carbon particles over the 48 hour period following injection into body wall tunic. Analysis of variance of mean per cent of hyaline amoebocytes with carbon as a function of

TABLE III

The concentration of blood cells (x 10^3) per cubic millimeter of blood and tunic with the levels of significance (P) of difference in mean concentration based on a t-test. Tunic_B indicates tunic of the body wall, tunic_S indicates tunic of the stolon

		M.M.	D.V.C.	H.A.	G.A.
Blood	Mean	0.54	0.38	3.14	2.26
	S.E.	0.10	0.09	0.58	0.42
Tunic_B	Mean	2.84	11.06	6.43	6.62
	S.E.	0.66	1.55	0.89	0.76
Tunic_S	Mean	123.18	47.50	45.32	47.50
	S.E.	23.99	9.62	8.96	9.62
Blood *versus* Tunic_B	*P*	0.02	0.001	0.02	0.001
Blood *versus* Tunic_S	*P*	0.01	0.01	0.001	0.01
Tunic_B *versus* Tunic_S	*P*	0.01	0.01	0.01	0.01

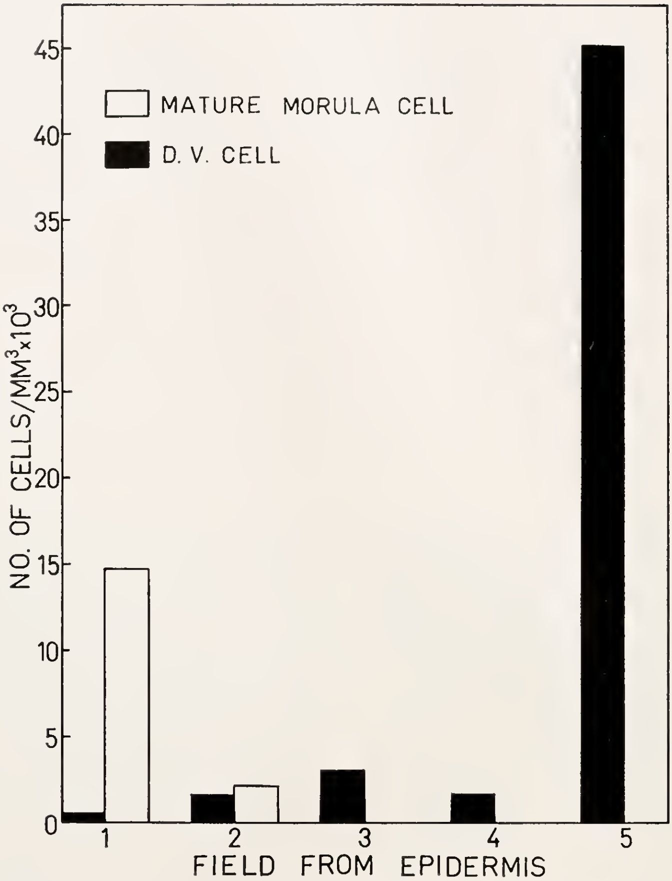


FIGURE 7. The concentration of mature morula and dispersed vesicular cells as a function of position in the tunic. Field 1 is at the epidermis and field 5 is at the external periphery of the tunic. Values reported are the mean concentrations for a sample of 5 animals with six replicate counts per animal.

time after injection gives a significant F ratio ($P < 0.01$). There is an increase in number of hyaline amoebocytes with carbon after injection with a maximum reached at 18 hours (Fig. 8). Mean comparisons of per cent of cells with carbon show that by 6 hours there are greater numbers of cells with particles than there are in controls, and this significantly higher number of cells with carbon is maintained over the 48 hour study period. The nature of the particles in the hyaline amoebocytes of the control studies is not known. However, hyaline amoebocytes often display some particulate or granular inclusions and hyaline amoebocytes which contained such particles were counted in the baseline controls to obviate misinterpretation in the phagocytosis studies.

IX. Tunic injury

Analyses of variance of the concentration of the four blood cell types in tunic at the site of injury as a function of day after injury reveal significant increases in the number of mature morula cells, including the *tunic vesicular cells* ($P <$ 0.005) and dispersed vesicular cells ($P < 0.005$). The maximum concentration of the two cells in the wound area is reached by the fifth day after injury (Fig. 9). By the third day after injury, there is a significantly higher concentration of mature morula cells than there is in control preparations. By the fifth day, the concentration of dispersed vesicular cells is significantly higher than controls. Fifteen days after injury, the concentration of dispersed vesicular cells remains as high as at five days after injury; however, the mature morula cell concentration has fallen. Neither the hyaline amoebocyte nor the granular amoebocyte increase significantly in the injury area over the period of study.

The injury to tunic abolishes the positional relationship of mature morula and dispersed vesicular cells. Within a day after injury, although there are higher

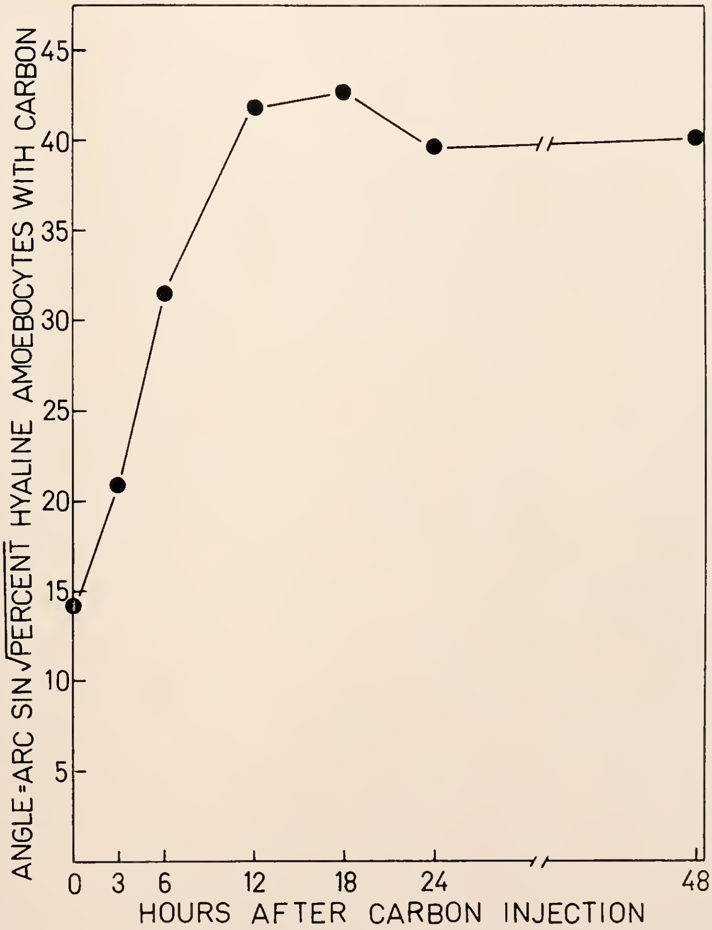


FIGURE 8. The per cent of hyaline amoebocytes with carbon particles as a function of time after carbon injection into body wall tunic. Each point represents the mean value for six animals.

levels of both cell types in the tunic, the cells are equally distributed between the inner and outer halves of the tunic injury area. By the tenth day after injury, there are significantly higher numbers of dispersed vesicular cells in the outer half of the tunic. By the fifteenth day, there is a significantly higher concentration of mature morula cells in the inner half of the injured tunic (Table IV).

As the mature morula cells and dispersed vesicular cells migrate into injured tunic, there are particular changes in both these cell types and the tunic material which they invade. The changes in the cells are essentially similar to those that take place upon migration into uninjured tunic. Initially, the mature morula cells congregate and break down at the edge of the wound. The lamination of the tunic matrix, which has been cut by the incision, is pinched together (Figs. 6C, 6D). After several days the matrix surrounding the edges of the wound is filled with a material which has staining properties similar to the mature morula

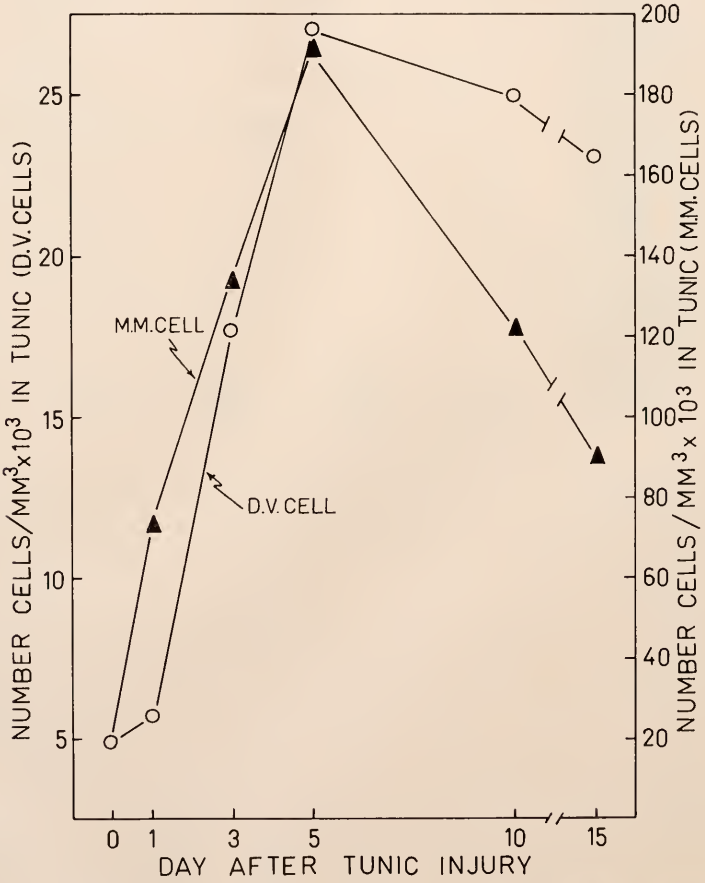


FIGURE 9. The concentration of mature morula cells, including *tunic vesicular cells,* and dispersed vesicular cells (× 10^3) per mm^3 of tunic as a function of day after injury. The ordinate on the right refers to the mature morula cells, that on the left to the dispersed vesicular cells. Each point represents the mean value for six animals.

Table IV

The significance levels of the differences in mean concentration of dispersed vesicular cells (D.V.C.) and mature morula cells (M.M.) per cubic millimeter between the inner (+) and outer (−) halves of the tunic as a function of time after injury. Also included are the significance levels of the differences in concentration of these cells in similar areas of uninjured tunic and in stolon at the half distance between the growing tip and the body wall of the animal.

	Cell type	
	M.M.	D.V.C.
Stolon	+0.01	−0.001
Uninjured tunic	+0.025	−0.005
Day after injury		
0	+0.001	N.S.
1	N.S.	N.S.
3	N.S.	N.S.
5	N.S.	N.S.
10	N.S.	−0.01
15	+0.01	−0.001

cells (Figs. 6C, 6D), and a great number of tunic vesicular cells are evident. Twenty days after injury, the external edge of the wound is covered with a dense staining cuticular material similar to the material covering the interpapillary areas of uninjured tunic. The fibrous material below the wound is lifted towards the wound and constricts there. The wound scar is denser and rougher than uninjured tunic and is not as heavily pigmented.

Discussion

It has been acknowledged generally that there is great variability in the concentration and differential distribution of blood cells in the ascidians both within and between species. There have been reports of variation in differential distribution as a function of position in the body of the tunicate, season of the year, reproductive condition, and detunication (George, 1926; Pérès, 1948a and 1948b; Millar, 1953; Cuenot cited by Andrews, 1962; Freeman, 1964). In some cases all of the blood cell types are represented in the tunic (Berrill, 1950) or only particular types are found there (Seeliger and Hartmeyer, 1911; Henze, 1913; Hecht, 1918; St. Hilaire, 1931; Pérès, 1948a, Endean, 1955b and 1961). Further, it has been stated that blood cells are located in specific areas of the tunic (Hecht, 1918; St. Hilaire, 1931; Das, 1936; Millar, 1953; Endean, 1961). Upon injury to tunic, increases in the number of blood cells in the tunic have been reported also (Hecht, 1918; St. Hilaire, 1931; Pérès, 1948b; Endean, 1961). There have been few estimations of the concentration of cells in blood or tunic (St. Hilaire, 1931; Endean, 1955a, 1955b, and 1960).

That the blood cells of the ascidians are present in areas other than the blood, such as the tunic, does not directly indicate that these areas are the sites of terminal function for the blood cells. In ascidians aggregation of blood cells in tunic could be due to a variety of factors: the tunic might be an aggregation area for senescent or dying cells or it might be one of many compartments accessible to wandering cells whose function lies elsewhere. On the other hand, the cells might have an intermediate rather than terminal function in tunic, such as trephocytosis (Liebman, 1947) or they might have a terminal function in the tunic whether degradative or synthetic.

To gain insight into possible roles that blood cells play in the tunic and in the blood requires primarily a definite recognizable categorization of cell types and a quantitative measurement of the number and kinds of cells in the blood. Consequent to this, measurements of the partition of cells into compartments and their localization in these compartments can be verified or negated statistically, so that some certitude can be attached to the fact that particular cell types are a) present in tunic, b) are present in tunic in numbers greater than, less than, or equal to blood concentration, and c) are localized or randomly distributed within the tunic. Some of the properties of these cells in tunic then can be investigated by quantifying their response in areas of rapid tunic growth or tunic injury. By comparing the kinds and numbers of cells in rapidly growing tunic and injured tunic with blood, the selective migration of particular cell types into tunic can be verified and distinctions between growth and traumatic phenomena can be made.

Some cell functions in the tunic can be investigated directly, for example phagocytosis, while others, at this stage, must be inferred. These inferences of blood cell function must take into account the structure and function of the system in which the cells aggregate, *i.e.*, the tunic. Consequent to such preliminary studies, hypotheses follow which can be tested by a variety of means such as histochemical, biochemical, or chemical analyses of the cells and tunic.

Unfortunately there has been no statistical analysis of the number and kinds of cells in blood, in tunic, or of the partition of cells between these two systems although there has been some numerical data reported which was derived from small sample sizes.

The morphology of the blood cells from ascidians has been investigated by a number of authors (Ohuye, 1936; George, 1939; Pérès, 1943; Andrew, 1962). In general, the blood cells of ascidians can be classified into three categories: vacuolar cells, amoebocytes, and lymphocytes of stem cells. The first category includes vanadocytes, ferrocytes, and their precursors (Webb, 1939; Endean, 1955a; 1960). The second category includes the phagocytes. The third category includes those undifferentiated cells which may differentiate into the first two categories of cells (George, 1939; Endean, 1955a; 1960), or divide before differentiation into the first two categories (Pérès, 1943; 1947), or initiate budding in certain colonial species (Freeman, 1964). The signet ring, compartment cell, immature morula, and mature morula cell (which is morphologically similar to the ferrocyte and contains iron) of *H. aurantium* belong to the first category. It must be noted, however, that not all morula-like cells in ascidians are metal bearing cells; in *Perophora viridis* there are reported to be two morula-like cells. The first of these is called a morula cell but it does not stain with osmium and is non-refractile in the living state. The second morula-like cell is referred to as a green cell; it has green refractile vacuoles in the living state and stains with osmium (George, 1926; Freeman, 1964). It has been proposed that the green cell is the vanadium bearing cell in *Perophora viridis* (George, 1926). The hyaline amoebocyte, granular amoebocyte, and dispersed vesicular cell would fall into the second category. The stem cell, the giant stem cell, and the stem cell with acidophilic vacuoles or granules belong to the third category of cells found in ascidians.

The quantitative information concerning the concentration of cells per mm^3 in blood or tunic is limited. In *H. aurantium,* the mean number (17,240) of cells per mm^3 is lower than that reported for other species of ascidian (Endean, 1955a; 1960, Freeman, 1964). The mature morula and immature morula cells of *H. aurantium,* make up approximately 30% of the circulating blood cells. This figure is lower than that reported for analogous cell types in other species (Endean, 1955a; 1960; and Freeman, 1964).

In *Pyura stolonifera,* the concentration of cells in the tunic, many of which are mature and immature morula cell analogues, has been estimated at 33,200 mm^3. Mature morula cell analogues are also present in the tunic of *Ascidia atra* (Hecht, 1918), *Phallusia mammillata* (Endean, 1961), and *Ascidia pygmaea* (Kalk, 1963). In *Phallusia*, deteriorated mature morula cell analogues are degraded to a bladder cell (tunic vesicular cell analogue) which are concentrated in the outer $\frac{3}{4}$'s of the tunic (Endean, 1961). The cell concentration in the tunic of *C. papillosa* is 240 per mm^2 (St. Hilaire, 1931). If these sections were cut at 10 microns, this would amount to 24,000 cells per mm^3 of tunic.

In *Halocynthia aurantium,* there are about 28,000 cells per mm^3 of body wall tunic. This figure is comparable with that for *Pyura* (Endean, 1955b) and *Cynthia papillosa* (St. Hilaire, 1931). The four cell types, mature morula, dispersed vesicular cell, hyaline amoebocyte, and granular amoebocyte are at significantly higher concentrations in the tunic of *H. aurantium* than they are in blood. The mature morula cell is concentrated in the basal layers of tunic adjacent to the epidermis and the dispersed vesicular cell is concentrated at the external periphery of the tunic. Both the mature morula cell and the dispersed vesicular cell undergo changes in the tunic which preclude their migration out of the tunic.

Migration of particular blood cell types into injured tunic of ascidians has been noted before (Hecht, 1918; St. Hilaire, 1931; Pérès, 1948b; Endean, 1961), but in none of the cited cases were there statistical evaluations of the injury response. Since it is difficult to standardize the degree of injury, and since there is great variation in the response of individual ascidians to injury, a careful statistical analysis of injured versus control data is necessary. Upon injury to the tunic of *H. aurantium*, only the mature morula cell and dispersed vesicular cell types are found in the injury area in significantly higher numbers than in controls. These two cell types then rapidly reassume their positional relationships in the tunic.

There is no increase in the number of hyaline amoebocytes in the injury area of tunic in *Halocynthia* over a 15 day period. The hyaline amoebocytes correspond to the macrophages of other authors (George, 1939; St. Hilaire, 1931; Endean, 1955a; 1960). In *Halocynthia*, the injection of carbon material into tunic material results in a graded increase in the number of hyaline amoebocytes with carbon particles over a 48 hour period. Since the blood was drawn from the ventral sinus rather than the site of carbon injection, the hyaline amoebocytes must be able to move in and out of the tunic. St. Hilaire (1931) has noted, that upon injection of carmine into general circulation, phagocytes would carry it into the tunic. Since the hyaline amoebocytes of *Halocynthia* appear to be freely mobile, the failure to note an increase in number of hyaline amoebocytes in injured tunic does not rule out the possibility of increased flux.

In summary then, there is statistical evidence from this work that in *Halocynthia* certain blood cell types have a characteristic localization and concentration within the tunic which suggests that they have a secretory and/or organizational function and that one of thet cell types in tunic is a phagocyte.

The tunic of the ascidians has been categorized generally as either fibrous or gelatinous (St. Hilaire, 1931); the tunic of *H. aurantium* would be considered fibrous. Lamination of tunic fibers is of widespread, but not general, occurrence in ascidians (St. Hilaire, 1931; Millar, 1953). St. Hilaire (1931) noted, based on a sample of three animals, an increase in number of laminae with increased size of *Cynthia papillosa*. There is a definite correlation ($r = +0.95$) between the number of laminae and wet weight of *Halocynthia aurantium*.

Lamination has been explained on the basis of sea water pressure acting during tunic secretion in *Cynthia papillosa* (St. Hilaire, 1931). *H. aurantium* does not have any gas space, and the interior of the animal is, for the most part, open to the sea via the atrial cavity. At the depth at which *Halocynthia* is found, the fluctuations of pressure due to tidal changes represent a small fraction of total pressure. Thus, the bands of laminae of diverse thickness cannot be explained on the basis of pressure.

These bands of thick and thin laminae may be accounted for by seasonal variation in reserve substances available for tunic formation. *H. aurantium* has one major spawning period, in the late spring and early summer (Smith, unpubl. obs.). Fluctuations of tissue metabolites as a function of gonadal cycle and season have been reported for several marine invertebrates (Barry and Munday, 1959; Nimitz and Giese, 1964).

The lamination, itself, is probably the result of the mature morula cell's contribution to the tunic. The tunic is not secreted from the epidermis as a sheet, but as single fibers. In the region of the body wall tunic where these fibers coalesce to form laminae, the mature morula cells aggregate and break down losing their characteristic staining properties. In the tunic of the body wall and in the tunic of laboratory grown stolon proximal to the body of the ascidian, regular lamination of the fibers is evident. At the growing tips of natural and laboratory grown stolon, the tunic material is quite thin, no lamination of fibers is obvious, and the mature morula cells are positioned at the peripheral edge of the tunic material. In the wall of natural stolon, the fibers are arranged irregularly. The function of the stolon is to form a hold-fast. Since the mature morula cells are located at the exterior edge of the tunic in the growing tip of stolon, the condensation of fibers would be determined by the contours of the substrate. In natural stolon, then, it would be expected that the fibers would assume the irregular contours of the substrate. In the body wall, and in the wall of laboratory grown stolon, the mature morula cells are located just peripheral to the epidermis, and there is no directional restriction of fiber condensation as there is with natural stolon. Consequently, the coalescence of fibers in these areas, through the mediation of the mature morula cells, will assume a regularity resulting in lamination.

That the mature morula cell's function is the condensation or coalescence of fibers in the tunic is further evidenced in injury repair studies. In this case, the initial reaction is a massive migration of mature morula cells to the external edges of the wound. In this position, the mature morula cells break down and there is a concurrent pinching together and coalescence of the edges of cut laminae. It has been suggested that mature morula cell analogues are capable of secreting tunicin in *Phallusia mammillata* and *Pyura stolonifera* (Endean, 1961; 1955b). Histochemical evidence does not support this hypothesis in the case of *Halocynthia aurantium* (Smith, 1969).

The mature morula cells decrease in relative number per unit of blood volume with increased weight of animal. They are concentrated in the body wall tunic in a restricted area adjacent to the epidermis. In this position, they degenerate which precludes the possibility of further differentiation or exit from the tunic. The decrease in relative concentration of mature morula cells may be a function of this positional relationship in the tunic. The aggregation of mature morula cells just above the epidermis resembles a surface. Since the surface and volume of a sphere or cylinder do not increase at the same rate, there could be a relative decrease in mature morula cells in respect to the volume of blood with increased size of ascidian, while a constant number of mature morula cells per unit tunic surface area is maintained.

The external surface of the ascidian tunic, particularly in the *Pyuridae,* often displays fibrous processes, spines, and cuticle structures (Berrill, 1950), and *Halocynthia* has both cuticle and spines. Kennedy (1966) has shown that a tunic cell in *Ascidia aspersa,* which may be analogous to the dispersed vesicular cell, will take up radioiodine and this cell is incorporated into the cuticle. It has been proposed that the cuticle and spines of *Cynthia papillosa* are composed of the same substance as the tunic matrix, but that the mode of deposition differs (St. Hilaire, 1931). St. Hilaire, (1931) suggested further that the dispersed vesicular cell analogue in *Cynthia* does not make a substantial contribution to the substance of the spines and cuticle. This does not appear to be true, however, for *Halocynthia.*

In natural stolons, laboratory grown stolons, body wall tunic, and injured tunic two properties stand out; a high concentration of dispersed vesicular cells at the periphery of the tunic material, and the presence of spines or cuticle in these areas. The spines are spaced at regular intervals over the surface of body wall tunic, irrespective of size of animal. Therefore, as the ascidian grows, there must be an absolute increase in the number of spines. The most obvious region for the initiation of the growth of new spines is from the interpapillary surface between older spines. These regions are particularly rich in dispersed vesicular cells.

Both the cuticle and spines have analogous staining properties which differ from those of the tunic matrix (Smith, 1969). Further, the blood dispersed vesicular cells have some histochemical properties in common with spine material and the vesicles of the dispersed vesicular cell stain for protein (Smith, 1969). The dispersed vesicular cells at the edge of tunic material display characteristics which could indicate that they are expended functionally: vesicles are small and more difficult to stain, the cytoplasm has lost its ability to take up stains, and the nucleus appears necrotic.

It is proposed that the dispersed vesicular cells have the function of forming cuticle and spines. Spines will be elaborated upon completion of the initial growth process which may involve mature morula cells. In natural stolon, there is a surface-to-substrate contact which may inhibit the elaboration of spines. In injury repair studies, initially, the edge of the wound mimics the tips of growing stolons in that there is a cuticle present and both mature morula and dispersed vesicular cells are present and not positionally separated. Given sufficient time, the edge of the wound might elaborate spines. An analogous situation is found in laboratory grown stolon. At the growing tip no spines are present, but there is a graded development of spines along the length of stolon with fully developed spines at the juncture of the body wall and stolon. In all cases, including the surface of interpapillary areas of body wall tunic, a cuticle is secreted and dispersed vesicular cells are present.

The dispersed vesicular cells also decrease in relative concentration in blood with increased weight of animal and accumulate in tunic material as a surface aggregation. The arguments presented to explain the decrease in relative number of mature morula cells in the blood also are valid for the dispersed vesicular cells (see above).

The relative number of hyaline amoebocytes in blood increase with increased weight of ascidian. There are no obvious areas or organs in *H. aurantium* which display large aggregations of deteriorating hyaline amoebocytes, such as is found for the dispersed vesicular cells and mature morula cells in the tunic. The increase in relative numbers of hyaline amoebocytes in blood cannot be explained as a reciprocal function of the decrease in other cell types because there is no difference in the absolute concentration of blood cells per unit blood volume as a function of weight. However, if the circulating life span of hyaline amoebocytes exceeds that of the mature morula cells, dispersed vesicular cells, and their precursors, there might be an accumulation of hyaline amoebocytes with time as reflected in increased weight of animal.

The granular amoebocyte is an enigma. It does not possess staining characteristics which are common to any of the tunic structures (Smith, 1969). It does not change in relative number with change in weight of ascidian. This may be accepted as an argument that its function is relative to a given volume of blood rather than a target organ or area. It is possible that it is a coagulocyte, or has a lysosomal function but there is no definite evidence for this.

The stem cell in *H. aurantium* agrees morphologically with the lymphocyte category of other authors. It is accepted generally that the lymphocyte type gives rise to other blood cell types in ascidians (George, 1939; Pérès, 1943; Endean, 1955a; 1960). This investigation does not offer evidence which would negate this hypothesis.

It has been suggested that in *Phallusia mammillata* (Endean, 1960) and *Pyura stolonifera* (Endean, 1955a), the histogenetic pathway for the development of the vanadocyte and ferrocyte, which are analogous to the mature and immature morula cells of *H. aurantium*, is from the lymphocyte (stem), to the signet ring cell, to the compartment cell, to the vanadocyte or ferrocyte. In *H. aurantium*, the signet ring cells, compartment cells, immature morula cells, and mature morula cells stain for the presence of iron as do the ferrocyte and its vacuolated precursors in *Pyura stolonifera* (Endean, 1955a). This study does not offer evidence which would contradict Endean's scheme (1955a; 1960) for metal-bearing blood cell differentiation.

The author wishes to thank Dr. P. A. Dehnel of the University of British Columbia for his support and for critically reading the manuscript.

SUMMARY

1. Then morphological blood cell types are recognized in *Halocynthia aurantium*. The mature morula cells, dispersed vesicular cells, stem cells, and stem cells with acidophilic vacuoles or granules decrease in differential distribution with increased weight of animal. The hyaline amoebocyte increases in differential distribution with increased weight of animal.

2. There is no significant difference in total blood cell concentration per cubic millimeter of blood as a function of weight of animal.

3. The mature morula cells, dispersed vesicular cells, hyaline amoebocytes, and granular amoebocytes are at significantly higher concentrations in the tunic of the body wall and stolon than they are in the blood. The mature morula cell is concentrated in the tunic just peripheral to the epidermis and the dispersed vesicular cell is concentrated at the external limits of the tunic. The hyaline amoebocyte is a phagocyte.

4. Upon injury to the tunic, the mature morula cells and dispersed vesicular cells increase significantly in the wound area over a 15 day period. The positional relationships of these cells are rapidly reinstated in the tunic.

5. The tunic consists of three components: epidermis, fibrous matrix, and cuticle with spines. The matrix consists of fibrous laminae which increase in number with increased weight of animal.

6. The mature morula cells, immature morula cells, compartment cells, and signet ring cells contain protein bound iron.

LITERATURE CITED

ANDERSON, R. S., 1969. Cellular responses of tunicates to foreign bodies. *Amer. Zool.*, **9**: 1116–1117.

ANDREW, W., 1962. Cells of the blood and coelomic fluid of tunicates and echinoderms. *Amer. Zool.*, **2**: 285–297.

BARRY, R. J., AND K. A. MUNDAY, 1959. Carbohydrate levels in *Patella*. *J. Mar. Biol. Ass., U. K.*, **38**: 81–95.

BERRILL, N. J., 1950. *The Tunicata*. Ray Society, London, 354 pp.

BERTRAND, D., 1950. The biogeochemistry of vanadium. *Bull. Amer. Mus. Nat. Hist.*, **94**: 407–455.

CULLING, C. F. A., 1963. *Handbook of Histopathological Technique*. [2nd. Ed.] Butterworths, London, 553 pp.

DALES, R. P., 1965. Iron compounds in the heart body of the terebellid polychaete *Neoamphitrite figulus*. *J. Mar. Biol. Ass. U. K.*, **45**: 341–351.

DAS, S. M., 1936. On the structure and function of the ascidian test. *J. Morphol.*, **59**: 589–601.

DECK, J. D., E. D. HAY AND J. REVEL, 1966. Fine structure and origin of tunic of *Perophora viridis*. *J. Morphol.*, **120**: 267–280.

ENDEAN, R., 1955a. Studies of the blood and tests of some Australian ascidians. I. Blood of *Pyura stolonifera*. *Aust. J. Mar. Freshwater Res.*, **6**: 35–59.

ENDEAN, R., 1955b. Studies of the blood and tests of some Australian ascidians. II. The test of *Pyura stolonifera*. *Aust. J. Mar. Freshwater Res.*, **6**: 139–156.

ENDEAN, R., 1960. The blood cells of the ascidian, *Phallusia mammillata*. *Quart. J. Microscop. Sci.*, **101**: 177–197.

ENDEAN, R., 1961. The test of the ascidian, *Phallusia mammillata*. *Quart. J. Microscop. Sci.*, **102**: 107–117.

FREEMAN, G., 1964. The role of blood cells in the process of asexual reproduction in the tunicate *Perophora viridis*. *J. Exp. Zool.*, **156**: 157–185.

George, W. C., 1926. The histology of the blood of *Perophora viridis* (Ascidian). *J. Morphol. Physiol.,* **41** : 311–331.

George, W. C., 1939. A comparative study of the blood of the tunicates. *Quart. J. Microscop. Sci.,* **81** : 391–431.

Gomori, G., 1941. Observations with differential stains on human Islets of Langerhans. *Amer. J. Pathol.,* **17** : 395–406.

Hecht, S., 1918. The physiology of *Ascidia atra. J. Exp. Zool.,* **25**: 229–299.

Henze, M., 1911. Untersuchungen über das Blut der Ascidien. I. Mitteilung. Die Vanadiumverbindung der Blutkörperchen. *Hoppe-Seyler's Z. Physiol. Chem.,* **72**: 494–501.

Kalk, M., 1963. Intracellular sites of activity in the histogenesis of tunicate vanadocytes. *Quart. J. Microscop. Sci.,* **104**: 483–493.

Kennedy, R. K., 1966. The distribution and nature of iodine compounds in ascidians. *Gen. Comp. Endocrinol.,* **7** : 500–511.

Levine, E. P., 1962. Studies on the structure, reproduction, development, and accumulation of metals in the colonial ascidian *Eudistoma ritteri* Van Name. *J. Morphol.,* **111** : 105–137.

Li, J. C. R., 1964. *Statistical Inference, Vol. I.* Edward Brothers, Inc., Ann Arbor, Michigan, 658 pp.

Liebman, E., 1947. The trephocytes and their functions. *Experientia,* **3** : 442–451.

Millar, R. H., 1953. *Ciona, L.M.B.C. Memoirs XXXV.* Liverpool University Press, Liverpool, 122 pp.

Nimitz, M. A., Sr., and A. C. Giese, 1964. Histochemical changes correlated with reproductive activity and nutrition in the chiton, *Katharina tunicata. Quart. J. Microscop. Sci.,* **105** : 481–495.

Ohuye, T., 1936. On the coelomic corpuscles in the body fluid of some invertebrates. III. The histology of the blood of some Japanese ascidians. *Sci. Rep. Tohoku Imp. Univ.,* **13** : 25–35.

Pearse, A. G. E., 1961. *Histochemistry, Theoretical and Applied.* [2nd Ed.]. J. and A. Churchill, Ltd., London, 998 pp.

Pérès, J. M., 1943. Recherches sur le sang et les organes neuraux des tuniciers. *Ann. Inst. Oceanogr.,* **21** : 229–359.

Pérès, J. M., 1947. Intérdependance du sang, du mesenchyme et de la tunique chez les ascidies. *Bull. Mus. Hist. Natur. Marseille,* **7** : 83–87.

Pérès, J. M., 1948a. Recherches sur la genèse et la régénération de la tunique chez *Clavelina lepadiformis* Muller. *Arch. Anat. Microsc. Morphol. Exp.* **37** : 230–260.

Pérès, J. M., 1948b. Recherches sur la genèse et al régénération de la tunique chez *Ciona intestinalis L. Bull. Inst. Oceanogr.,* **936**: 1–12.

Ries, E., 1937. Die Tropfenzellen und ihre Bedeutung für die Tunicabildung bei *Clavelina. Wilhelm Roux Arch. Entwicklungsmech. Organismen.,* **137** : 363–371.

St. Hilaire, K., 1931. Morphogenetische Untersuchungen des Ascidienmantels. *Zool. Jahrbuch. Abt. Anat. Ontog.,* **54** : 455–608.

Seeliger, O., and R. Hartmeyer, 1911. *Dr. H. G. Bronn's Klassen und Ordnungen des Tier-Reichs. Bd. III, Suppl., Tunicata.* C. F. Winter'sche Verlagshandlung, Leipzig, 1773 pp.

Smith, M. J., 1969. Studies of the blood cells and tunic of the ascidian, *Halocynthia aurantium* (Pallas). *Ph.D. thesis, University of British Columbia,* 143 p.

Snedecor, G. W., 1956. *Statistical Methods.* [5th Ed] Iowa State University Press, Ames, Iowa, 534 pp.

Steel, R. G. D., and J. H. Torrie, 1960. *Principles and Procedures of Statistics with Special Reference to the Biological Sciences.* McGraw-Hill, New York, 481 pp.

Van Name, W. G., 1945. The North and South American ascidians. *Bull. Amer. Mus. Natur. Hist.,* **84**: 1–476.

Webb, D. A., 1939. Observations on the blood of certain ascidians, with special reference to the biochemistry of vanadium. *J. Exp. Biol.,* **16**: 499–522.

Webb, D. A., 1956. The blood of tunicates and the biochemistry of vanadium. *Publ. Sta. Zool. Napoli,* **28**: 273–288.

Reference: *Biol. Bull.,* **138**: 379–388. (June, 1970).

THE BLOOD CELLS AND TUNIC OF THE ASCIDIAN *HALOCYNTHIA AURANTIUM* (PALLAS). II. HISTOCHEMISTRY OF THE BLOOD CELLS AND TUNIC[1]

MICHAEL J. SMITH[2]

Department of Zoology, University of British Columbia, Vancouver 8, Canada

In the first paper of this series (Smith, 1969), the morphology of the tunic and blood cells of the ascidian *Halocynthia aurantium* (Pallas) was described. Of the ten blood cell types recognized in this species, four are involved in the tunic: the mature morula cell, the dispersed vesicular cell, the hyaline amoebocyte, and the granular amoebocyte. The mature morula cell and dispersed vesicular cell appear to be intimately involved with tunic growth and repair. The hyaline amoebocyte is a phagocyte. Suggestions have been made concerning the function of these blood cell types in the tunic and their interrelationships based on quantitative considerations of the distribution of these cells within and between animals.

It has been suggested that blood cells can secrete tunic (Herdman, 1899; Ries, 1937; Endean, 1955a, 1955b, 1960, and 1961), or that blood cells have an organizational, rather than secretory, function in the tunic (St. Hilaire, 1931; Das, 1936), or that the tunic is secreted primarily by the epidermis (Deck, Hay and Revel, 1966).

There have been numerous reports of the presence of cellulose in ascidian tunic (reviewed by Seeliger and Hartmeyer, 1911; Spence and Richards, 1940; Tsuchiya and Suzuki, 1963) and more recently acid mucopolysaccharides and protein have been reported (Hall and Saxl, 1960 and 1961; Bierbauer and Vagas, 1962; Godeaux, 1963).

Many of the suggestions for the origin of the tunic are based on histochemical analogy between the tunic and epidermis or tunic and blood cells. There is reason to doubt the specificity of the histochemical and fixation methods of some of the older work in light of recent reports on the nature and specificity of particular histochemical methods (Hale, 1957; Curran, 1961; Quintarelli, 1968).

This study was undertaken to ascertain the histochemical composition of the tunic, epidermis, and blood cells involved in the tunic of *H. aurantium* in order to clarify the origin of tunic and the function of the blood cells in the tunic.

MATERIALS AND METHODS

The ascidians were collected and held in the laboratory as previously described (Smith, 1969).

---

[1] This work was supported by grants from the National Science Foundation (#GN 4358) and the National Research Council of Canada (T88) to Dr. P. A. Dehnel, University of British Columbia.

[2] Present address: Department of Zoology, University of Nebraska, Lincoln, Nebraska. This work is a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. in the Department of Zoology, University of British Columbia.

For routine fixation buffered formalin with cetyl pyridinium bromide in distilled water (Culling, 1963) was used. Other fixatives employed were 5% formalin in sea water, Bouin's, Baker's, Carnoy's (Culling, 1963), and N-methyl morpholine with cyanuric chloride in methanol (Goland, Nicholas and Kazimieras, 1967). Tissues were fixed for four hours in Carnoy's and for twenty-four hours in all other fixatives.

After fixation, tissues were washed in water, dehydrated in graded ethanols, cleared in benzene, embedded in Para-plast (M.P. 55.6° C), and 8 micron serial sections were cut on a Spencer microtome.

Animals less than two grams wet weight were used for histochemical preparations. The use of small whole animals facilitates the simultaneous histochemical examination of both blood cells and tunic within a single animal. All histochemical tests were performed on tissues from at least two animals and replicate slides were made from each animal.

For the detection of carbohydrates, the following procedures were employed: alcian blue with neutral red, alcian blue with periodic Schiff reaction, periodic Schiff reaction (P.A.S.), Gomori's aldehyde fuchsin (Culling, 1963), toluidine blue, methylene blue extinction test with veronal acetate buffers at approximately 0.50 pH increments from pH 1.63 to pH 9.05, Hale's dialyzed iron method (colloidal iron) (Pearse, 1961), and azure A at pH 1.5 and 4.0 (Szirmai, 1963). Some sections were methylated or methylated and saponified prior to staining with alcian blue, colloidal iron, aldehyde fuchsin, toluidine blue, and azure A (Culling, 1963). Controls for the P.A.S. reaction were filter paper and cotton gauze as well as known mucinous structures in the ascidians.

Proteins were detected histochemically by the Millon reaction, the dihydroxy-dinaphthyl-disulphide (DDD) method for sulfhydryls of Barnett and Seligman, and the mercury bromphenol blue method of Bonhag (Pearse, 1961). The presence of basic, histone-like proteins was determined with Bierbrich's scarlet (Spicer, 1962).

R.N.A. was stained by the methyl green-pyronin method with parallel control sections incubated in ribonuclease (Nutritional Biochemicals) (Pearse, 1961).

In order to elucidate the chemical structure of tunic, as measured by histochemical means, some tunic was treated with either chemical agents or enzymes to ascertain their effect on tunic staining properties. Prior to such treatments tunic was washed with distilled water, ethanol, acetone, and ether and then dried for 48 hours *in vacuo* over phosphorous pentoxide and sodium hydroxide.

Dried tunic was submitted to one of two chemical treatments: 8 hours hydrolysis in 6 *N* HCl under anaerobic conditions at 100° C, or 24 hours in 1 *N* HCl at 8° C followed by 36 hours in 1 *N* NaOH at 100° C. The second method parallels the procedure for the purification of lobster chitin (Hackman, 1954).

Tunic was treated with pronase, hyaluronidase, pronase followed by hyaluronidase, or a combination of papain and trypsin. The pronase (California Biochemicals) incubation mixture consisted of 0.5 mg of enzyme per mg of dried tunic in 0.15 *M* phosphate buffer at pH 8.0. The buffer was 0.005 *M* in $CaCl_2$. The digestion period was 48 hours (Barker and Young, 1966; Inoue and Yosizawa, 1966). Bovine testes hyaluronidase (California Biochemicals) medium consisted of 0.2 *M* phosphate buffer at pH 6.8 with an enzyme to substrate ratio (by weight) of 1 : 10 (Dorfman, 1955). Tunic was treated with hyaluronidase for 14 days at 37° C as was tunic which had been pretreated with pronase for 48 hours. Sections of tunic also were incubated with hyaluronidase for 24 hours according to the method of Culling (1963).

Tunic was also digested with papain (Schiller, Slover and Dorfman, 1961) followed by digestion with trypsin (Schiller, Mathews, Jefferson, Ludowieg and Dorfman, 1954).

Results and Discussion

1. Histochemistry of blood cells in circulation

Conventional methods of staining for neutral and acidic polysaccharides indicate that the mature morula (Table I), immature morula cells, compartment cells, and signet ring cells do not contain these substances. The vesicles of the dispersed vesicular cells will react positively to P.A.S. only when fixed with N-methyl morpholine and cyanuric chloride (Table I). Both the mature morula cells and dispersed vesicular cells are involved in the tunic (Smith, 1969). The mature morula cells, which accumulate iron (Smith, 1969), are analogous to vanadocytes and ferrocytes (Webb, 1939; Endean, 1955a, and 1960), but unlike these cells do not stain for carbohydrate (Endean, 1955a and 1960). The dispersed vesicular cell of *Halocynthia* may be analogous to the tunic cell of *Ascidia aspersa* which takes up radioiodine and which is incorporated into the cuticle (Kennedy, 1966).

Table I

The histochemical reactions of the mature morula and dispersed vesicular cells (D.V.) in both the blood and tunic are summarized. The following convention was utilized to express intensity of staining: − negative, ± faint positive, + definitely positive, ++ strong positive, and +++ intense positive. Alpha refers to an alpha metachromasia or orthochromasia. The reactions noted are the reactions of the vacuoles of the mature morula cell and the vesicles of the despersed vesicular cell.

Reaction	Mature Morula		D.V. Cell	
	Blood	Tunic	Blood	Tunic
P.A.S.	−	−	+	+
Alcian blue	−	−	+ to −	−
Toluidine blue	−	± to − (alpha)	−	−
Colloidal iron	−	−	+	−
Azure A pH 1.5	−	−	−	−
Azure A pH 4.0	±	++ to ±	+	−
Aldehyde fuchsin	+	++ to ±	+ to −	−
Methylene blue extinction (pH)	9.05	9.05	5.00	5.35
Methyl green pyronin (RNA)	−	−	± to −	−
DDD Sulfhydryl	−	−	−	−
Bierbich's scarlet	± to −	−	+	−
Mercury bromophenol blue	+	+	+	−
Millon's	±	±	±	±

It has been suggested that in *Halocynthia* the histogenetic pathway for the differentiation of the mature morula cell type passes from the signet ring cell through the compartment cell and immature morula to the mature morula cell (Smith, 1969); this sequence parallels the hypothesis of Endean (1955a and 1960) concerning the histogenesis of analogous cell types in *Pyura stolonifera* and *Phallusia mammillata.* The consistent positive reaction of the vacuoles of the signet ring, compartment, immature morula, and mature morula cells to mercury bromphenol blue, Biebrich's scarlet, and iron stains (Smith, 1969) indicates that these cells are chemically related. The presence of accumulated iron and protein is a property which they share with the ferrocyte of *Pyura stolonifera* (Endean, 1955a).

In the blood both the hyaline amoebocytes and granular amoebocytes stain with P.A.S. (Table II). The staining is diffuse in hyaline amoebocytes, but concentrated in the granules of the granular amoebocyte. The hyaline amoebocyte is a phagocyte (Smith, 1969), but no function has been assigned to the granular amoebocyte although it, and the hyaline amoebocyte, are present in the tunic of *Halocynthia* (Smith, 1969). Both of these cells are negative to acid mucopolysaccharide stains.

Of the blood cells, the stem cell, the hyaline amoebocyte, some signet ring cells, stem cells with acidophilic vacuoles and granules, granular amoebocytes, and some dispersed vesicular cells have ribonuclease labile affinity for pyronin in the methyl green-pyronin technique for R.N.A. If R.N.A. content, as judged by pyronin affinity, can be used as a criterion of synthetic activity, then the signet ring cell probably represents the intermediate between the stem cell with acidophilic vacuoles and granules and the compartment cell.

II. Histochemistry of the mature morula and dispersed vesicular cells in the tunic

The histochemical reactions of the mature morula and dispersed vesicular cells in the tunic are summarized in Table I.

Although the mature morula cell retains its negative reaction to polysaccharide stains, it does display a variety of reactions in the tunic, as contrasted with the

Table II

The histochemical reactions of the hyaline amoebocyte and granular amoebocyte in the blood of H. aurantium. *The reaction of the cells are indicated as follows:* + *definitely positive,* ± *faintly positive,* − *negative. Alpha refers to an orthochromatic reaction*

Reaction	Hyaline Amoebocyte	Granular Amoebocyte
P.A.S.	+	+
Alcian blue	−	−
Toluidine blue	± (alpha)	−
Aldehyde fuchsin	−	+ to −
Methylene blue extinction (pH)	5.00	5.00
Methyl green pyronin (R.N.A.)	+ to −	+
Millon's	±	±

blood, which indicate that some chemical change is taking place in the cell prior to its degradation to the *tunic vesicular cell.* For example, the mature morula cells in the tunic show a variety of reactions to azure A at pH 4.0. This variability is also true when the cells are stained with aldehyde fuchsin. The *tunic vesicular cells,* which are degraded mature morula cells in the tunic (Smith, 1969), only rarely have an affinity for the histochemical stains used in this study. The mature morula cell in the tunic differs from the blood mature morula in its staining reactions to aldehyde fuchsin, Biebrich's scarlet, azure A at pH 4.0 (Table I), and Perl's method for iron (Smith, 1969). The *tunic vesicular cell* differs from the tunic mature morula cell in that it displays occasional reactions with colloidal iron, shows a variety of reactions to aldehyde fuchsin although it rarely takes the stain, and it has lost its reactivity to Millon's reagent and mercury bromphenol blue. These reactions indicate that a chemical change is taking place in the sequence: blood mature morula cells, tunic mature morula cells, and *tunic vesicular cells.* This change appears to include a loss of protein material.

The dispersed vesicular cell in the tunic also displays changes in stain affinity which indicate that it is losing or depositing some material at the periphery of the tunic (Table I). Although the vesicles retain a P.A.S. reaction when fixed with cyanuric chloride and N-methyl morpholine, these vesicles lose their reactivity to alcian blue, colloidal iron, azure A at pH 4.0, never display a positive R.N.A. reaction when the cell is in the tunic, and lose their affinity for the protein stains (Table I). There also appears to be an increase in the M.B.E. point of the vesicles of this cell in the tunic.

Vacuolar blood cells in the tunics of *Clavelina lepadiformes* (Ries, 1937), *Pyura stolonifera* (Endean, 1955b), *Phallusia mammillata* (Endean, 1961), and *Ciona intestinalis* (Pérès, 1948b) contain a carbohydrate material as demonstrated by histochemical methods and are reported to be involved in tunic formation. Endean (1955b and 1961) states that the function of the vanadocyte and ferrocyte is tunicin secretion. In *H. aurantium,* although the mature morula cells deteriorate in the tunic as do the vanadocyte and ferrocyte, the mature morula cells consistently display negative reactions for either neutral or acidic polysaccharides. This suggests that the mature morula cells do not contribute to tunic carbohydrate. The staining reactions of the mature morula do indicate protein, and this protein stain affinity is lost by the time this cell degrades to a *tunic vesicular cell.* These reactions indicate that the mature morula cell may deposit protein in the tunic. The localization of the mature morula cells in body wall tunic just above the epidermis suggests that the function of these cells may be organizational in the sense that they contribute directly or indirectly to the fibers of tunic as they are secreted from the epidermis, organizing these fibers into definite layers or laminae (Smith, 1969).

III. Histochemistry of the tunic

There have been numerous reports of positive histochemical reaction of tunic for neutral carbohydrates or cellulose (St. Hilaire, 1931; Ries, 1937; Pérès, 1948a and 1948b; Endean 1955b and 1961; Deck *et al.*, 1966). It has been noted also that tunic is metachromatic (Pérès, 1948a and 1948b), and more recently it has been reported that the tunic contains acidic polysaccharides or mucopolysaccharides (Endean, 1955a and 1961; Bierbauer and Vagas, 1962). The tunic matrix of *H. aurantium* gives a very weak (always less than cotton gauze or filter paper controls) to negative reaction with P.A.S. and stains as an acid mucopolysaccharide (Table III. The fibrous matrix of the tunic also has a positive histochemical reaction for protein. Chemical analyses of *H. aurantium* reveal that the tunic is approximately 50% protein and 50% carbohydrate by weight (Smith, 1969).

The cuticle and cuticular spines of *H. aurantium* show a positive reaction for some protein stains (Table III). Kennedy (1966) has reported that the cuticle of *Ascidia aspera* is scleroprotein. The negative reaction for sulfhydryl groups of the cuticular spines does not exclude the possibility of disulfides since the sections were not treated with a reducing agent prior to the histochemical reaction.

The reaction of the tunic matrix to acid mucopolysaccharide stains is more complex than is immediately apparent (Table III). Upon methylation and saponification there is a return of staining intensity with azure A at pH 1.5 to a level greater than the methylated section, but not to the level of control sections. Since carboxyl groups should be protonated at pH 1.5 and sulfate groups should be removed by methylation technique (Culling, 1963), the resurgence of staining

TABLE III

The histochemical recations of the three tunic components, matrix fibers, spines, and epidermis. The following convention was used to indicate intensity of staining: – negative, ± faint positive, + definitely positive, ++ strong positive, +++ intense positive. Alpha, beta, and gamma refer to orthochromatic color, violet, and red metachromasia, respectively.

Reaction	Matrix Fibers	Spines	Epidermis
P.A.S.	± to –	–	–
Methylene blue extinction (pH)	pH 1.63	pH 4.28	pH 1.63
Toluidine blue	+++ gamma	+ alpha	+ beta
Azure A pH 1.5	+++	±	++
Methylated	± to –	–	–
Saponified	++ to +	–	+
Azure A pH 4.0	+++	+	++
Methylated	±	+	+ to ±
Saponified	++	+	+
Aldehyde fuchsin	+++	±	++
Methylated	++	–	–
Saponified	++	–	+
Alcian blue	+++	–	+
Methylated	++ to +	–	+
Saponified	+	–	+
Colloidal iron	+++	–	+
Methylated	++ to +	–	±
Saponified	++ to +	–	±
DDD Sulfhydryl	–	–	±
Biebrich's scarlet	+	–	± to –
Millon's	±	±	±
Mercuric bromphenol blue	± to –	+	–
Methyl green pyronin (RNA)	–	–	+

intensity can be explained on the basis of incomplete methylation, exceptionally strongly dissociated carboxyl groups, or the presence of an uncharacterized substrate. The methylation period was 24 hours, so it is difficult to accept incomplete methylation as an alternative.

It has been reported that in *Perophora viridis* the epidermis will take up tritiated glucose which eventually appears in the tunic matrix, but that the vanadocyte does not accumulate the label (Deck *et al*, 1966). These observations and the histochemically analogous staining reactions of epidermis and tunic matrix in *Halocynthia* (Table III) are consistent with the hypothesis that the epidermis is the major tunic secreting system. This is reinforced by the fact that the epidermis of *Halocynthia* stains for the presence of R.N.A., as would be expected of a metabolically active tissue, whereas the mature morula and immature morula cells never display the presence of R.N.A.

IV. The effect of chemical and enzymic treatment on tunic histochemistry

The effects of hyaluronidase, pronase, and trypsin/papain digestion on tunic structure are summarized in Table IV. The epidermis and blood cells are destroyed by the preparative procedures. Morphologically there does not appear to be any change in matrix structure as a result of either proteolytic or hyaluronidase digestion. In *Pyura stolonifera* hyaluronidase treatment causes a disarrangement of fibrillar structure (Endean, 1955b), but in *Halocynthia* the orderly arrangement of fibers into laminae is unaffected by hyalurinidase digestion.

The cuticular spines undergo a limited degradation of structure upon hyaluronidase treatment and a greater degradation when treated with proteolytic enzymes (Table IV).

Acid, and acid followed by base, treatment of the tunic of *Halocynthia* completely destroys the cuticular spines, but there is no change in the orderly laminar structure of the fibrous matrix.

It is understandable that the tunic would furnish a negative P.A.S. reaction

TABLE IV

The effects of various experimental treatments on the structure and Alcian Blue-P.A.S. reactivity of the tunic fibers and spines. The following abbreviations are used: A.B. alcian blue, P.A.S. Periodic Acid Schiff reaction, Hy'ase hyaluronidase, N. C. no change in the structure, N.R. no reaction, C.D. completely degraded, and P.D. partially degraded.

	Tunic Fibers			Tunic Spines		
	A.B.	P.A.S.	Remarks	A.B.	P.A.S.	Remarks
Treatment						
Control	+++	± to −	N.C.	—	—	N.C.
Tunicin prep.	±	−	N.C.	N.R.	N.R.	C.D.
6 *N* HCl 8 hours	+	−	N.C.	N.R.	N.R.	C.D.
Hyaluronidase	++	−	N.C.	−	−	P.D.
Pronase/Hy'ase	++	−	N.C.	−	−	P.D.
Trypsin/papain	++	−	N.C.	−	−	P.D.

if the carbohydrate component of tunic were masked by protein. Acid, acid and base, or proteolytic enzyme treatment should remove protein and reveal available P.A.S. reactive sites on the carbohydrate. However, subsequent to such treatment the tunic retains its overall negative reaction to P.A.S. (always less than cotton gauze or filter paper controls). Such chemical or enzymic treatment of tunic does decrease its affinity for alcian blue (Table IV) with the greatest decrease caused by acid and base treatment. If the carbohydrate component of tunic is a cellulose-like polymer, a possible explanation for the negative P.A.S. reaction after such chemical or enzymic digestion is extensive micelle formation due to hydrogen bonding between adjacent linear carbohydrate polymers (Cowling, 1963; Jayme and Lang, 1963) which denies access to the histochemical reagent molecules. This does not seem likely after strong acid and base treatment or if we consider that the P.A.S. reaction depends on a prior oxidation of the substrate with periodic acid. If the substrate molecule is not cellulose, then the failure of the P.A.S. technique can be explained on the basis of a lack of available vicinal diglycols which could be due to extensive cross-linking, deoxy sugars, or resistant glycosidic linkages through sugar carbons not involved in polymeric linkage.

These histochemical analyses of blood cells and tunic, coupled with quantitative evidence of discrete aggregation areas for the mature morula and dispersed vesicular cells in the tunic (Smith, 1969), allow certain hypotheses concerning the blood cells, tunic, and the function of the blood cells in the tunic.

This study does not support the hypothesis that the iron bearing cell contributes carbohydrate to the tunic, although it may contribute protein. The only tissue in *Halocynthia* which displays staining reactions that might indicate carbohydrate secretion is the epidermis. Further, the staining reactions of epidermis are analogous to those of the tunic matrix fibers which again would indicate that the epidermis is the tunic secreting tissue. However, the function of the mature morula cell must be closely allied to tunic formation since it displays aggregation and degeneration in the tunic just above the epidermis (Smith, 1969). The mature morula cell could be serving one of several functions in this position: it may be contributing protein to the tunic fibers as extruded from the epidermis, it may be instrumental in the formation of laminae or fibrous layers in the tunic (Smith, 1969), or it may be performing the latter function through the former. The histochemical reactions of the tunic matrix and cuticle and the resistance of tunic matrix to P.A.S., even after chemical or enzymatic treatment, indicate that the tunic is a complex chemical entity. If cellulose does exist in the tunic of *Halocynthia* it is, at least, heavily substituted. Preliminary investigations reveal that the tunic of *Halocynthia* contains significant amounts of hexosamine, is 50% protein, that serine and glucosamine are involved in the protein-polysaccharide linkage, and that the carbohydrate component of tunic has only a limited susceptibility to cellulase (Smith, in preparation). Consequently, the question of the composition of ascidian tunic, and more particularly the protein-polysaccharide composition, is still open.

The author wishes to thank Dr. P. A. Dehnel of the University of British Columbia for his support through this study and Dr. R. Meints of the University of Nebraska for critically reading the manuscript.

SUMMARY

1. The similar staining properties of the vacuolar blood cells of *Halocynthia aurantium* indicate the relationship of the cell types constituting this group.

2. The mature morula cells and dispersed vesicular cells of *Halocynthia* do not stain for the presence of polysaccharides, but do stain for protein.

3. Changes in the staining properties of mature morula and dispersed vesicular cells between blood and tunic indicate that these cells contribute protein to tunic structures.

4. The tunic matrix and epidermis show stain affinities characteristic of acid mucopolysaccharide material.

5. The tunic matrix fibers display a consistent negative reaction to the P.A.S. technique. Neither enzymatic nor chemical treatment of tunic increases the reactivity of the fiber to P.A.S. reagent.

LITERATURE CITED

BARKER, S. A., AND N. M. YOUNG, 1966. Isolation of hyaluronic acid from human synovial fluid by pronase digestion and gel filtration. *Carbohyd. Res.*, **2**: 49–55.

BIERBAUER, J., AND E. VAGAS, 1962. Histological and histochemical examination of tunic formation on the *Ciona intestinalis*. *Acta Biol. Acad. Sci. Hung.*, 12 (Suppl. 4): 29.

COWLING, E. B., 1963. Structural features of cellulose that influence its susceptibility to enzymatic hydrolysis, pp. 1–32. *In:* E. T. Reese, Ed., *Advances in Enzymic Hydrolysis of Cellulose and Related Materials.* Pergamon Press, London.

CULLING, C. F. A., 1963. Handbook of Histopathological Technique. 2nd ed. Butterworths, London, 553 pp.

CURRAN, R. C., 1961. The histological demonstration of connective tissue mucopolysaccharides. In, *Biochemistry of Mucopolysaccharides of Connective Tissue. Biochem. Soc. Symp. 20,* pp. 24–38. Edited by F. Clark and J. K. Grant. Cambridge Univ. Press, London.

DAS, S. M., 1936. On the structure and function of the ascidian test. *J. Morphol.*, **59**: 589–601.

DECK, J. D., E. D. HAY AND J. REVEL, 1966. Fine structure and origin of tunic of *Perophora viridis*. *J. Morphol.*, **120**: 267–280.

DORFMAN, A., 1955. Mucopolysaccharidases, pp. 166–173. *In:* S. P. Colowick and N. Kaplan, Eds., *Methods in Enzymology.* Academic Press, New York.

ENDEAN, R., 1955a. Studies of the blood and tests of some Australian ascidians. I. Blood of *Pyura stolonifera*. *Aust. J. Mar. Freshw. Res.*, **6**: 35–59.

ENDEAN, R., 1955b. Studies of the blood and tests of some Australian ascidians. II. The test of *Pyura stolonifera*. *Aust. J. Mar. Freshw. Res.*, **6**: 139–156.

ENDEAN, R., 1960. The blood cells of the ascidian, *Phallusia mammillata*. *Quart. J. Microscop. Sci.*, **101**: 177–197.

ENDEAN, R., 1961. The test of the ascidian, *Phallusia mammillata*. *Quart. J. Microscop. Sci.*, **102**: 107–117.

GODEAUX, J. E. A., 1963. Integument of tunicata. *Proc. VI Int. Congr. Zool.*, **16**(1): 16.

GOLAND, P., G. NICHOLAS AND K. V. KAZIMIERAS, 1967. Cyanuric chloride and N-methyl morpholine in methanol as a fixative for polysaccharides. *Stain Technol.*, **42**: 41–51.

HACKMAN, R. H., 1954. Studies on chitin. I. Enzymic degradation of chitin and chitin esters. *Aust. J. Biol. Res.*, **7**: 168–178.

HALE, A. J., 1957. The histochemistry of polysaccharides. *Int. Rev. Cytol.*, **6**: 193–264.

HALL, D. A., AND H. SAXL, 1960. Human and other animal cellulose. *Nature*, **187**: 547–550.

HALL, D. A., AND H. SAXL, 1961. Studies of human and tunicate cellulose and their relation to reticulin. *Proc. Roy. Soc. London Series B*, **155**: 202–217.

HERDMAN, W. A., 1899. *Ascidia. Liverpool Marine Biology Committee Memoirs*, **1**: 1–60.

INOUE, S., AND Z. YOSIZAWA, 1966. Purification and properties of sulfated sialopolysaccharides isolated from pig colonic mucosa. *Arch. Biochem. Biophys.* **117**: 257–265.

Jayme, G., and F. Lang, 1963. Cellulose solvents. *Methods in Carbohydrate Chem.*, **3**: 75–83.

Kennedy, G. R., 1966. The distribution and nature of iodine compounds in ascidians. *Gen. Comp. Endocr.*, **7**: 500–511.

Pearse, A. G. E., 1961. *Histochemistry, Theoretical and Applied.* [2nd ed.] J. and A. Churchill Ltd., London, 998 pp.

Pérès, J. M., 1948a. Recherches sur la genèse et la régénération de la tunique chez Clavelina lepadiformes Muller. *Arch. Anat. Microsc. Morphol. Exp.*, **37**: 230–260.

Pérès, J. M., 1948b. Recherches sur la genèse et la régénération de la tunique chez Ciona intestinalis L. *Bull. Inst. Oceanogr.*, **936**: 1–12.

Quintarelli, G., 1968. Methods for the histochemical identification of acid mucopolysaccharides: A critical evaluation, pp. 199–218. *In:* G. Quintarelli, Ed., *The Chemical Physiology of Mucopolysaccharides.* Little, Brown and Co., Boston.

Ries, E., 1937. Die Tropfenzellen und ihre Bedeutung für die Tunicabildung bei *Clavelina. Wilhelm Roux Arch. Entwicklungsmech. Organismen.*, **137**: 363–371.

Saint Hilaire, K., 1931. Morphogenetische Untersuchungen des Ascidienmantels. *Zool. Jahrbuch, Anat.*, **54**: 435–608.

Schiller, S., M. B. Mathews, H. Jefferson, J. Ludowieg and A. Dorfman, 1954. The metabolism of mucopolysaccharides in animals. I. Isolation from skin. *J. Biol. Chem.*, **211**: 717–724.

Schiller, S., G. A. Slover and A. Dorfman, 1961. A method for the separation of acid mucopolysaccharides: Its application to the isolation of heparin from the skin of rats. *J. Biol. Chem.*, **236**: 983–987.

Seeliger, O., and R. Hartmeyer, 1911. *Dr. H. G. Bronn's Klassen und Ordnungen des Tier-Reichs.* Bd. III, Suppl., Tunicata. C. F. Winter'sche, Leipzig, 1773 pp.

Smith, M. J., 1969. Studies of the blood cells and tunic of the ascidian, *Halocynthia aurantium* (Pallas). *Ph.D. thesis, University of British Columbia*, 143 pp.

Spence, J., and O. W. Richards, 1940. Native cellulose in the ascidian *Phallusia nigra. Publ. Carnegie Inst.*, **517**: 163–167.

Spicer, S. S., 1962. Basic protein visualized histochemically in mucinous secretions. *Exp. Cell Res.*, **28**: 480–488.

Szirmai, J., 1963. Quantitative approaches in the histochemistry of mucopolysaccharides. *J. Histochem. Cytochem.*, **11**: 24–33.

Tsuchiya, Y., and Y. Suzuki, 1963. Biochemical studies of the ascidian, *Cynthia roretzi v. Drasche.* V. Tunicin in the test. *Tohoku J. Agr. Res.*, **14**: 39–43.

Webb, D. A., 1939. Observations on the blood of certain ascidians, with special reference to the biochemistry of vanadium. *J. Exp. Biol.*, **16**: 499–522.

Reference: *Biol. Bull.*, **138**: 389–396. (June, 1970).

SURVIVAL OF UNFED SQUID, *LOLIGO PEALEI*, IN AN AQUARIUM[1]

WILLIAM C. SUMMERS AND JOHN J. McMAHON

Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Literature dealing with laboratory studies of live squid usually includes a statement that attempts to rear or maintain squid in aquaria are unsuccessful. When stated, the survival of squid in captivity is most often given for the longest lived individual and predictive survival rates are not specified. A considerable body of current research (principally that dealing with squid "giant axons") depends on a reliable supply and short term maintenance of squid. Several aspects of squid biology lag for want of effective means to carry out lengthy laboratory studies. Preparatory to possible future studies on the maintenance of the squid, *Loligo pealei* (Lesueur, 1821), we have observed squid survival when subjected to standardized conditions. Quantitative data were obtained in an attempt to specify survivorship for comparison with other reports and for evaluation of certain environmental factors.

The most dramatic results of attempts to rear cephalopods were reported by Choe (1966), who succeeded in fostering a few individuals of three species for more than 100 days and one loliginid squid for over 40 days. Edward T. LaRoe (University of Miami, unpublished) has repeatedly reared *Sepioteuthis sepioidea* to near maturity. Adults of this species have been maintained in floating pens for two months and possibly longer (Arnold, 1965). Immature squid, *Loligo vulgaris,* were reported to survive captivity for several weeks (Bidder, 1950) and for two months (Tardent, 1962). Tardent (1962) stated that sexually mature *L. vulgaris* do not normally live more than two weeks in the Naples Aquarium. Bidder (1950) reported that *L. vulgaris* is less sensitive to handling than *Loligo forbesi* and quoted a Dr. Sereni in the opinion that *L. pealei* at Woods Hole is far hardier and more resistant to handling than *L. vulgaris* at Naples. Roper (1965) reported a female *Doryteuthis plei* which survived 18 days in an aquarium, laying two egg masses in that time. Arnold (1962) gives the survival of sexually mature *L. pealei* as about five days and one instance of a male surviving for 17 days. These survival times refer to the longevity of the last remaining individual. In all of the references cited, squid were fed regularly and provided with running seawater. Only references dealing with loliginid squid have been cited because the survival of other families of cephalopods may not be comparable due to differences in life habits and ecological tolerances.

---

[1] This research was supported by NIH contract 69-2009 to the Marine Biological Laboratory and by the Marine Biological Laboratory. The use of facilities and field units provided by the Systematics-Ecology Program of the Marine Biological Laboratory is gratefully acknowledged.

Methods

Squid survival studies were conducted from mid-May through September, 1969, at the Marine Biological Laboratory, Woods Hole, Massachusetts. Seawater temperatures in the experimental aquarium were near ambient for the nearby squid habitats and ranged from 14.4 to 23.2° C; the maximum temperature occurred in late August. Live squid were captured by a floating fishtrap and by otter trawl nets The fishtrap was located off Quissett Harbor in Buzzards Bay and was operated intermittently during the summer months by Mr. Charles L. Wheeler of the Bureau of Commercial Fisheries, Biological Laboratory (Woods Hole). Trap hauls were made approximately every other day, usually in the morning. Otter trawl procedures were essentially the same as previously described (Summers, 1968). The #35 otter trawl cited was modified by replacement of the rubber rollers on the foot rope with a chain, exchange of the cod end liner for one with a stretched mesh dimension of 1.3 cm and use of trawl doors weighing 140 kg each. Most of the trawling was done with a 45–65 Long Island Sound balloon trawl made by George W. Wilcox Company, Mystic, Connecticut. The 45–65 trawl was narrower and taller than the #35 trawl, but utilized the same mesh dimensions, fittings and trawl doors. Trawling was restricted to midday tows in 10 to 30 m depths within a 20 km radius of Woods Hole (*i.e.*, Buzzards Bay, Vineyard Sound and western Nantucket Sound).

Trap caught squid were dip netted into tubs in a trap skiff and transferred to a cylindrical wooden tank of approximately 900 liter capacity aboard the R/V BLUEBACK. Running seawater was pumped directly into this tank. The BLUEBACK normally returned to the dock in 20 to 30 minutes where the remaining live squid were promptly dip netted into tubs and wheeled to the experimental tank on a cart. A final selection was made for apparently healthy squid before initiating the experimental procedure. Trawl caught squid were separated from the remainder of the catch in a periodically flushed, seawater filled tub and transferred to a conical fiberglass tank of approximately 400 liter capacity on the deck of the R/V A. E. VERRILL. Running seawater was pumped directly into this tank. The conical shape effectively dampened water movements during vessel operations. Dead and moribund squid were periodically removed because the VERRILL made from two to four trawl tows of 15 to 30 minutes each and sometimes took as long as 1½ hours to return to the dock. Handling at the dock was similar to the procedure for trap caught squid.

The experimental aquarium was kindly furnished by Mr. C. O. Schweidenback of the Marine Biological Laboratory. It was a rectangular, paraffin coated, concrete tank with inside dimensions of 98 by 138 cm and 65 cm deep. An overflow drain located near one corner kept the water depth at 45 cm giving a seawater volume of approximately 600 liters. A small piece of netting was anchored opposite the drain as a substratum for egg deposition. A copious supply of seawater was provided by an inflow hose located at the center of the tank, just below the water surface. A corrugated, green fiberglass cover with a single opening for the seawater inlet was fitted over the tank. During the summer months an airstone was placed in a vacant corner of the tank for additional aeration. Constant illumination was provided by a 75 watt spotlight set 25° off the vertical and shown directly on the center of the tank cover from a distance of

140 cm. This produced a uniform, diffuse light in the tank which was little affected by room lights and activities near the tank. The experimental tank was occasionally drained and hosed down.

Constant illumination was chosen for these runs because light was required for observations and because *L. pealei* is demersal in daylight hours (Summers, 1969 and unpublished results). The value of this choice was demonstrated in an aborted run when, by mistake, the tank was left uncovered with the light off for a few minutes. Several squid jumped out of the tank and those remaining were found swimming actively.

Experimental runs were made by placing a group of squid in the tank and observing survival for up to ten days at a time. Squid were checked at least once a day and usually three times daily; dead squid and the major portion of any fresh egg mass were removed and the water temperature was recorded. The number, mantle length, sex and maturity of dead squid were noted with pertinent observations at each inspection. No attempt was made to duplicate the initial numbers of squid in the tank from run to run because the supply of healthy animals varied and strict replicate experiments were impossible with one tank.

Squid characteristics varied with both season and method of capture. Sex ratios were close to one-to-one in all cases. Trawl caught squid ranged in size from 9 to 28 cm dorsal mantle length with median sizes of approximately 15 cm throughout the study period. Sexually immature individuals were not common among trawl caught squid except for one group in mid-August. Trap caught squid ranged in size from 5 to 28 cm mantle length with an overall median size of 8 cm. The median size of trap caught squid decreased through the study period and, with the exception of the first trap haul in mid-June, these squid were predominantly sexually immature. Thus, the two methods of capture appear to have sampled different portions of the squid population except early in the summer when the mature squid come close inshore to breed (see Verrill, 1882; Summers, 1968 and 1969).

Squid in these experimental runs were purposely not fed for a number of reasons. We felt that feeding might stimulate competition and promote tank contamination. Attempts to feed the squid would produce additional disturbances and could not readily be regulated so that every squid received a share of the food. The logistics of catching and holding live food organisms were other considerations in our decision. Finally, the value of feeding was a significant question in itself. Bidder's reports on the rapid digestion of squid (1950 and 1966) led us to believe that they might quickly starve and we were prepared to change our experimental procedure if necessary. On the other hand, we were interested in the survival of the squid over the first few days in captivity and chose not to feed them for simplicity in experimental design.

RESULTS

Twelve experimental runs were continued until the last squid died or for a period of at least one week. Eight runs used trawl caught squid and four, trap caught squid; these were interspersed and randomly ordered according to availability. Experimental runs with trawl caught squid were generally begun with larger numbers of animals because trawl catches were larger than trap catches.

Survival of squid was averaged for the last observation in every 24 hour period for all runs resulting from a single method of capture. Figure 1 shows how the mean survival decreased with time and that the relative variation (standard deviation divided by the mean) increased with time for both trawl and trap caught squid. The mean survival of squid is shown on an absolute scale because the numbers of squid per run were kept within practical laboratory levels and there were insufficient runs, in our opinion, to justify the use of a relative scale. The observed mean survival of squid appears to be proportional to the number of squid in the tank and similar for both methods of capture. The survival rate does not vary appreciably with time in the tank within one week. Least square fit proportional survival curves are shown as lighter lines in Figure 1, these correspond closely to a survival rate of 71% per day (*i.e.,* 50% every two days). More exactly, it was 70.2% per day for trawl caught squid and 71.5% per day for trap caught squid.

Breeding activity and subsequent egg deposition is thought to cause rapid mortality in several species of cephalopods and is especially well documented for *Loligo opalescens* (McGowen, 1954; Limbaugh and Shepard, 1957; Hobson, 1965; Fields, 1965). Thus, survival of immature squid versus breeding, sexually mature squid as well as survival of males versus females in the latter group is of interest. The similarity of relative survival rates for trawl caught squid (sexually mature and breeding) and trap caught squid (sexually immature) shown in Figure 1 indicates that no major differences exist between these groups over a period of one

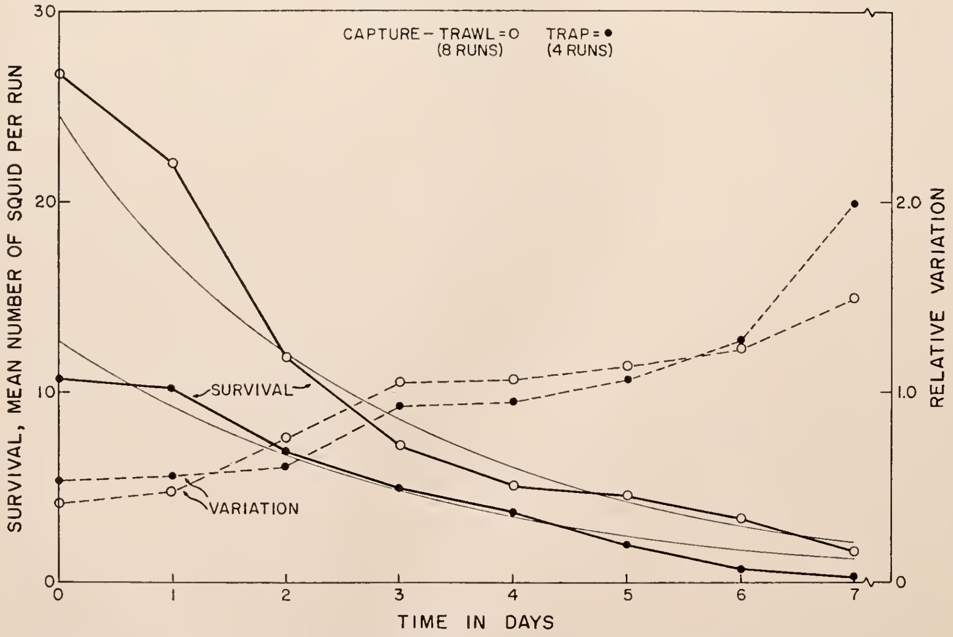


FIGURE 1. Mean survival of unfed squid, *L. pealei*, in an aquarium over a period of one week. Dashed lines represent the relative variation (standard deviation divided by the mean). Lighter lines are least square fit survival curves for the data and have proportional survival rates of 70.2% per day for trawl caught squid and 71.5% per day for trap caught squid.

week. Observations of dead or moribund squid associated with egg masses as reported for *L. opalescens* generally have no counterpart in *L. pealei* in either our field or laboratory experience.

Average mortalities illustrating sex and size factors are shown for selected runs employing trawl caught squid in Figure 2. The mortality of female squid shown in Figure 2A is based on four runs in which the numbers of sexually mature males and females were approximately equal. These data are contrasted with 49% of the total (unsexed) mortality (corresponding to the overall proportion of females in the runs). Use of a chi-square test failed to demonstrate significant differences between the observed and proportional mortalities of female squid over the period of one week.

Possible differential mortality of squid as a function of size was of concern in view of the literature on squid maintenance previously cited. The mortality of squid larger than the median size for each run (*i.e.*, larger than about 15 cm dorsal mantle length) shown in Figure 2B is based on seven runs containing predominantly sexually mature squid. These data are contrasted with 42% of the total mortality (corresponding to the overall proportion of larger squid in the runs). Use of a chi-square test failed to demonstrate significant differences between the observed and proportional mortalities of larger squid over the period of one week.

The results given above do not show effects related to water temperature; this varied almost 9° C during the experimental period, but never as much as 2° C

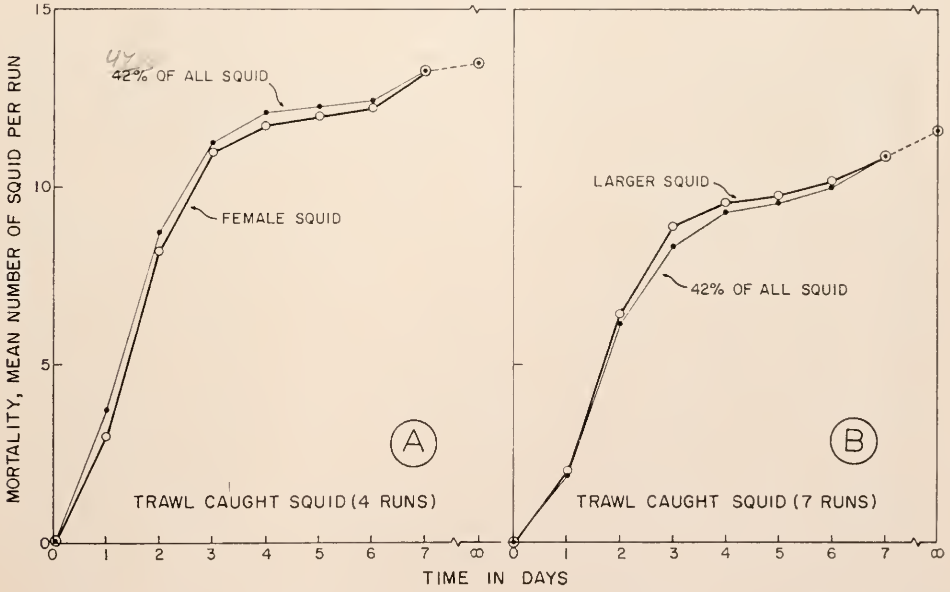


FIGURE 2. Mean mortalities of sexually mature, trawl caught squid, *L. pealei*, for selected runs over a period of one week. In Figure 2A, the mortality of female squid is contrasted with 49% of the total mortality (corresponding to the proportion of females) in four runs having approximately equal numbers of males and females. In Figure 2B, the mortality of larger squid (larger than the median mantle length) is contrasted with 42% of the total mortality (corresponding to the proportion of larger squid) in seven runs. Neither contrast is significant through the use of chi-square tests.

during any one run. In general, changes in water temperature were gradual and the animals were not subjected to water temperatures widely different from those occurring in nearby squid habitats. Although no optimum water temperature is implied, the three numerically most successful survivorships occurred at temperatures between 19.6 and 22.3° C. One run with an initial number of 40 squid and temperatures of 19.4 to 20.0° C, however, had no survivors on the fourth day.

Some notion of the probable course of any particular run was gained by an empirical evaluation of the initial survival rate. The observed number of survivors was compared with a calculated number based on the initial number of squid and the average survival rate of 71% per day. A survival trend was considered positive when the observed number was larger than the calculated value rounded to the nearest whole number. Five of the six runs with large initial numbers (21–40 squid) showed agreement in trends for both two and six day survivals. These six runs were equally divided positive and negative on the sixth day. Five of the six runs with small initial numbers (4–16 squid) had positive trends at two days, but agreement in trends for both two and six day survivals occurred in only one of the six runs. Eight of the twelve runs had negative survival trends on the sixth day. (In fact, five of the twelve runs had reached 100% mortality by the end of the sixth day.) Squid survived a full six days in runs with initial numbers of 39 (9 survivors), 30 (8 survivors), 30 (8 survivors), 16 (2 survivors), 31 (1 survivor), 21 (1 survivor) and 8 (1 survivor).

Discussion

The results presented here offer little that will optimize the maintenance of the squid, *L. pealei,* by way of selecting for a certain source, size or sex. Water temperatures between 14.4 and 23.2° C appear to be unimportant for laboratory survival. Survival for several days is numerically best with a large initial number of squid. Short term survival (*e.g.,* two days) is relatively better with small initial numbers of animals.

It was anticipated that survival would be influenced by several independent sources of mortality and that some of these would produce distinctive indentifiable trends. We anticipated that natural mortality from aging and related causes excluding predation would act at a low, constant rate which would be insignificant in our results. Our experimental method subjected squid to starvation and we expected a delayed, dramatic mortality during the run. In fact, no such mortality was observed even in runs which lasted ten days or longer. Although cannibalism of dead or dying squid was apparent in some cases, we cannot conclude that it was ever of sufficient quantity to sustain the surviving squid. We consider it unlikely that starvation was a major cause of mortality over a period of one week.

Persistent capture trauma cannot be eliminated as a cause of mortality in our experiments. Although the squid were carefully selected at the beginning of each experimental run, it is possible that some of them suffered an early death as a result of capture or handling injuries. We expected that this effect would result in a high initial mortality. We have noted that the proportion of squid surviving trawl capture appears to be inversely related to the quantity of the total catch and especially sensitive to the presence of rough surfaced invertebrates, sharks and fishes in the catch. We could not correlate laboratory survival of trawl caught squid with capture conditions and believe that the selection procedure effectively minimized the carryover of capture injuries. Furthermore, trap caught squid should have been free from capture damage, but not possible handling injuries. Figure 1 shows that the initial survival of trawl and trap caught squid followed the same relative pattern, therefore, we cannot credit capture trauma as a major cause of mortality.

Confinement of squid in the experimental tank promoted possible injury through unnatural and continual interaction with mechanical barriers. We anticipated that resulting mortalities would occur randomly with time and in proportion to the crowding of animals in the tank. We thought that crowding would increase the general level of activity and increase the probability that squid would injure themselves on the tank walls. Mortalities would follow injuries by some time which could not be specified, but which was considered variable and brief compared to the experimental run. Thus, we expected that confinement mortality would be roughly independent of confinement time and proportional to the number of squid. The observed results readily fit this model and we accept it as an important element among the causes of mortality in aquarium held squid.

The proportional survival model has several counterparts in familiar physical systems. Examples include radioactive decay and the kinetics of a first order chemical reaction. The response of an electronic lag network or mechanical spring dashpot system to step inputs are also analogous. Such transient phenomena are commonly described in control system theory with Laplace transformations. In examples where discrete units make up the particular assemblage under consideration, these are treated as statistically identical, each with an intrinsic probability for the subject event irrespective of all others. Although we recognize differences between the units in our study (individual squid), we have not succeeded in demonstrating that these are significant in laboratory survival. Hence, the proportional survival model is appropriate until separate causes of mortality can be isolated. The survival model can be expressed in the following formula: $n = n_0(R)^t$. Where n is the instantaneous number of surviving squid, n_0 is the initial number of squid, R is the average, daily survival rate (0.71 in our experiments) and t is the time in days. This formula is a special case of a more general class of equations having the form: $n = n_0(e^{-kt})$. Where k is a rate constant defined as follows: $k = \log_e(1/R)$.

Our results compare with those reported by Arnold (1962 and personal communication). Using four to ten, sexually mature *L. pealei* (mantle lengths of 15 to 25 cm) per run in a tank approximately 50% the capacity of ours, Arnold reported an average survival of about five days. Assuming his average run began with a middle number of squid (seven), that one squid (on an average) survived to the end of the fifth day and that survival rates in his runs were uniformly proportional, one can calculate a daily survival rate of 68% per day from his data. This is close to the 71% per day which we observed and may indicate that feeding (in Arnold's experiments) does not improve the short term survival rate.

The authors wish to acknowledge the many people who have assisted in various phases of this work. We are indebted to Mr. Charles L. Wheeler for fishtrap specimens and to the crew of the R/V A. E. Verrill for their patience in the tedious process of capturing live squid. We are especially indebted to Mr. Michael A. Soukup, a graduate student at the University of Massachusetts, who worked with us during the summer months. Mr. Soukup regularly participated in the fishtrap operation, shared in the periodic checking of the experimental tank and performed a number of other tasks which augmented this study. Drs. William J. Adelman, Jr. and Lawrence R. McCloskey read and criticized the manuscript.

SUMMARY

1. This paper reports the survival rate of unfed squid, *Loligo pealei*, in a 600 liter aquarium with running sea water and constant illumination.

2. Squid captured by a floating fishtrap and by otter trawl nets survived at the average rate of approximately 71% per day over a period of one week apparently independent of time in the tank, method of capture, water temperature, size or sex.

3. Initial numbers of squid between four and forty did not greatly alter the relative results. The early results, in conjunction with the survival rate given above and the initial number of squid, were useful in empirically evaluating the probable course of an experimental run.

4. It is possible that observed mortalities resulted from crowding and mechanical confinement. Other anticipated causes of mortality (*i.e.*, capture trauma and starvation) were not obvious in our results.

LITERATURE CITED

Arnold, J. M., 1962. Mating behavior and social structure in *Loligo pealii*. *Biol. Bull.*, **123**: 53–57.

Arnold, J. M., 1965. Observations on the mating behavior of the squid *Sepioteuthis sepioidea*. *Bull. Mar. Sci.*, **15**: 216–222.

Bidder, A. M., 1950. The digestive mechanism of the European squids, *Loligo vulgaris, Loligo forbesi, Alloteuthis media* and *Alloteuthis subulata*. *Quart. J. Microscop. Sci.*, **91**: 1–43.

Bidder, A. M., 1966. Feeding and digestion in cephalopods, pp. 97–122. *In:* K. M. Wilbur, and C. M. Yonge, Eds., *Physiology of Mollusca, Volume II*, Academic Press, New York.

Choe, Sang, 1966. On the eggs, rearing, habit of the fry and growth of some cephalopods. *Bull. Mar. Sci.*, **16**: 330–348.

Fields, W. G., 1965. The structure, development, food relations, reproduction and life history of the squid *Loligo opalescens* Berry. *Fish. Bull., California Fish and Game*, **131**: 1–108.

Hobson, E. S., 1965. Spawning in the Pacific Coast squid, *Loligo opalescens*. *Underwater Natur.*, **3**: 20–21.

Lesueur, C. A., 1821. Description of several new species of cuttlefish. *J. Acad. Natur. Sci. Philadelphia*, **2**: 86–101.

Limbaugh, Conrad, and F. P. Shepard, 1957. Submarine Canyons, pp. 633–640. *In:* J. W. Hedgpeth, Ed., *Treatise on Marine Ecology and Paleoecology, Volume 1.* Geological Society of America, New York.

McGowen, J. A., 1954. Observations of the sexual behavior and spawning of the squid, *Loligo opalescens*, at La Jolla, California. *California Fish and Game*, **40**: 47–54.

Roper, C. F. E., 1965. A note on egg deposition by *Doryteuthis plei* (Blainville, 1823) and its comparison with other North American loliginid squids. *Bull. Mar. Sci.*, **15**: 589–598.

Summers, W. C., 1968. The growth and size distribution of current year class *Loligo pealei*. *Biol. Bull.*, **135**: 366–377.

Summers, W. C., 1969. Winter population of *Loligo pealei* in the mid-Atlantic Bight. *Biol. Bull.*, **137**: 202–216.

Tardent, Pierre, 1962. Keeping *Loligo vulgaris* in the Naples Aquarium. *Bull. Inst. Oceanogr. Monaco*, Spec. No. **1A**: 41–46.

Verrill, A. E., 1882. Report on the cephalopods of the northeastern coast of America. *Rept. U. S. Comm. Fish.*, (Part 7) **1879**: 211–455.

INDEX

A

ACTH, effect on sloughing frequency of normal and thyroidectomized *Anolis carolinensis* of, 129.

Acclimation to freshwater by the earthworm, 247.

Acid mucopolysaccharides in tunic, 379.

Adaptation to (hyperbaric) oxygen by annelids, 235.

Aequipecten irradians, reproductive physiological variation in latitudinally separated populations of, 56.

AHMED, M. AND A. K. SPARKS. Chromosome number, structure and autosomal polymorphism in the marine mussels *Mytilus edulis* and *Mytilus californianus*, 1.

α-ecdysone and cyasterone, inactivation by fleshfly larvae and silkworm pupae of, 326.

α-ecdysone in silkworm pupae, distribution and metabolism of, 115.

ALVARADO, R. H. See T. H. DIETZ, 247.

Amino acids, translocation and utilization of by starfish, 14.

Amphiura chiajei, maturation of the gonads and seasonal cycle of the planktonic larvae of, 262.

ANDERSON, D. G. See J. R. RICE, 334.

Anemone, sea, burrowing behavior of, 316.

Annual reproductive cycle of *Amphiura chiajei* Forbes, 262.

Annual reproductive cycle of bay scallops, 56.

Anolis carolinensis, normal and thyroidectomized, effect of ACTH on sloughing frequency of, 129.

Antheraea polyphemus pupae, distribution and metabolism of α-ecdysone in, 115.

Aquarium, survival of unfed squid in, 389.

Arbacia punctulata, division times of whole eggs and haploid and diploid half-eggs of, 184.

Ascidians, blood cells and tunic of, 354.

Autoradiography of gut mucous cells in Echinoidea, 286.

Autoradiography of starfish, 14.

Axial units of flatworm spermatozoa, 219.

B

Barnacle nauplii, function of the frontolateral horns and horn glands of, 211.

Behavior and movements of weakly electric fishes, 200.

Biosynthesis of crassin acetate in *Pseudoplexaura porosa*, 334.

Blood cells and tunic of ascidians, 354, 379.

Bugula stolonifera, genetics of, 138.

Burrowing behavior of the sea anemone, 316.

C

CALDER, D. R. Hydroid and young medusa stages of *Dipurena strangulata* (Hydrozoa, Corynidae), 109.

Cell division rate of sea urchin eggs, 184.

Cephalopoda, laboratory maintenance of squid, 389.

Cercariae, marine, of the Woods Hole Massachusetts region, 66.

Cesium-137 gamma rays, effect on regeneration in *Tubularia* of, 194.

CHERBAS, L. AND P. CHERBAS. Distribution and metabolism of α-ecdysone in pupae of the silkworm *Antheraea polyphemus*, 115.

CHERBAS, P. See L. CHERBAS, 115.

CHIU, K. W. AND W. G. LYNN. The effect of ACTH on the sloughing frequency of normal and thyroidectomized *Anolis carolinensis* (Iguanidae, Lacertilia), 129.

Chlamydomonas eugametos, effect of concanavalin A upon the mating-type reaction of, 88.

Chlamydomonas moewusii, effect of concanavalin A upon the mating-type reaction of, 88.

Chloride transport in the osmotic and ionic regulation in the earthworm, 247.

Chromatophores in the prawn, 26.

Chromosome number and structure in *Mytilus*, 1.

Cirripedia nauplii, function of the frontolateral horns and horn glands of, 211.

Cnidaria, *Dipurena strangulata*, developmental stages of, 109.

Coelenterate behavior, 316.

Coelomic fluid composition in the earthworm, 247.

COLE, K. S. AND D. L. GILBERT. Jet propulsion of squid, 245.

Color changes in the prawn, 26.

Concanavalin A, effect on the mating-type reaction of chlamydomonads of, 88.
Control of ion balance in the earthworm, 247.
Crab, horseshoe, osmotic and ionic regulation in, 157.
Crassin acetate, isolation, localization and biosynthesis of in *Pseudoplexaura porosa,* 334.
Crassostrea virginica, influence of salinity and temperature on physiological activities of predators of, 35.
Crop fluid composition in the earthworm, 247.
Cyasterone and α-ecdysone, inactivation by fleshfly larvae and silkworm pupae of, 326.

D

Defense against predation by barnacle nauplii, 211.
Dietz, T. H. and R. H. Alvarado. Osmotic and ionic regulation in *Lumbricus terrestris* L., 247.
Diploid half-eggs of sea urchins, division times of, 184.
Dipurena strangulata, hydroid and young medusa stages of, 109.
Discharge characteristics of electric gymnotid fishes, 200.
Distribution and metabolism of α-ecdysone in pupae of *Antheraea polyphemus,* 115.
Diurnal movements of electric gymnotid fishes, 200.
Division times of whole, haploid and diploid half-eggs of sea urchins, 184.
Dosidicus gigas, jet propulsion of, 245.
Drills, oyster, influence of salinity and temperature on physiological activities of, 35.
Drosophila imaginal discs, metamorphosis of, 47.
Drosophila melanogaster, hormonal control of metamorphosis, 47.
Dugesia tigrina, role of illumination and temperature in the control of sexual reproduction in, 77.

E

Earthworms, osmotic and ionic regulation in, 247.
Ecdysone analogues, induction of metamorphosis in *Drosophila* imaginal discs by, 47.
Echinaster echinophorus, translocation and utilization of amino acids by, 14.
Echinoderm eggs, division times of, 184.
Echinodermata, comparative study of gut mucous cells in thirty-seven species of Echinoidea, 286.
Ectoprocta, phylum of, population genetics of marine species of, 138.
Effects of ACTH on skin histology in lizard, 129.
Effects of temperature and salinity on maturation of *Hydroides dianthus,* 306.
Eggs of sea urchins, division times of, 184.
Electric field, regenerative ability in ligated stems of *Tubularia* in an, 344.
Electric gymnotid fishes, diurnal movements and discharge characteristics of, 200.
Electro-location mechanism, use of by weakly electric fishes, 200.
Electron microscopy of flatworm spermatozoa, 219.
Electrophoresis of marine species of the phylum Ectoprocta, 138.
Environmental effects on Muricidae, 35.
Environmental induction of sexual reproduction in *Dugesia tigrina,* 77.
Environmentally induced or genetic variation of the bay scallop, 56.
Eupleura caudata, effects of salinity and temperature on feeding, reproductive and survival rates of, 35.
Extracellular volume of *Limulus,* 157.

F

Fecundity of *Hydroides dianthus,* 306.
Feeding rate of marine prosobranch gastropods, effects of salinity and temperature on, 35.
Fenaux, L. Maturation of the gonads and seasonal cycle of the planktonic larvae of the ophiuroid *Amphiura chiajei* Forbes, 262.
Ferguson, J. C. An autoradiographic study of the translocation and utilization of amino acids by starfish, 14.
Fingerman, M. Dual control of the leucophores in the prawn, *Palaemonetes vulgaris,* by pigment-dispersing and pigment-concentrating substances, 26.
Fishes, electric gymnotid, diurnal movements and discharge characteristics of, 200.
Flatworm microtubules, negative staining of, 219.
Fleshfly, inactivation of α-ecdysone and cyasterone by, 326.
Frontolateral horns and horn glands of barnacle nauplii, function of, 211.

G

Gametogenesis in *Aequipecten irradians,* 56.
Gametogenesis of *Hydroides dianthus,* 306.
Gamma rays, cesium-137, effect on regeneration in *Tubularia* of, 194.
Gastropods, feeding and reproductive rates of marine prosobranchs, 35.

Gel filtration on Sephadex LH-20 to separate dispersing and concentrating substances in white pigment of the prawn, 26.
Genetics, population, of marine species of the phylum Ectoprocta, 138.
Ghiselin, M. T. See N. D. Holland, 286.
Gilbert, D. L. See K. S. Cole, 245.
Gonad maturation of the planktonic larvae of *Amphiura chiajei,* 262.
Gooch, J. L. and T. J. M. Schopf. Population genetics of marine species of the phylum Ectoprocta, 138.
Gut mucous cells in thirty-seven species of Echinoidea, comparative study of, 286.

H

Habitat of *Limulus,* 157.
Halocynthia aurantium, blood cells and tunic of, 354, 379.
Haploid half-eggs of sea urchins, division times of, 184.
Heath, J. E. and R. K. Josephson. Body temperature and singing in the katydid *Neoconocephalus robustus* (Orthoptera, Tettigoniidae), 272.
Hemolymph circulation in the silkworm, 115.
Histochemistry of blood cells of *Halocynthia aurantium,* 379.
Histochemistry of gut mucous cells in Echinoidea, 286.
Holland, N. C. and M. T. Ghiselin. A comparative study of gut mucous cells in thirty-seven species of the class Echinoidea (Echinodermata), 286.
Hormonal control of metamorphosis in *Drosophila melanogaster,* 47.
Horn glands and frontolateral horns of barnacle nauplii, function of, 211.
Hydration of muscles of *Limulus,* 157.
Hydroid and young medusa stages of *Dipurena strangulata,* 109.
Hydroides dianthus, maturation of, 306.
Hydrozoa, life history of *Dipurena,* 109.

I

Illumination and temperature in the control of sexual reproduction of *Dugesia tigrina,* 77.
Inactivation of α-ecdysone and cyasterone by fleshfly larvae and silkworm pupae, 326.
Injury response in ascidians, 354.
Insect endocrinology, 47.
Interrupted doses in oxygen poisoning in annelids, 235.
Intracellular composition of muscle of *Limulus,* 157.
Ionic and osmotic regulation in *Limulus polyphemus,* 157.
Ionic regulation in *Lumbricus terrestris,* L., 247.
Isolation of crassin acetate in *Pseudoplexaura porosa,* 334.
Isopods, osmoregulatory capabilities in, 96.

J

Jet propulsion of squid, 245.
Josephson, R. B. See J. E. Heath, 272.

K

Katydid, body temperature and singing in, 272.

L

Larvae, planktonic, of *Amphiura chiajei,* maturation of the gonads and seasonal cycle of, 262.
Larval trematodes of the Woods Hole area, 66.
Leone, D. E. The maturation of *Hydroides dianthus,* 306.
Leucophores in the prawn dual control by pigment-dispersing and pigment-concentrating substances of, 26.
Life-history of *Dipurena strangulata,* 109.
Ligia occidentalis, osmoregulatory capabilities in, 96.
Ligia pallasii, osmoregulatory capabilities in, 96.
Light in planarian reproduction, 77.
Limulus polyphemus, osmotic and ionic regulation in, 157.
Liuzzi, A. See M. Slaughter, 194.
Localization of crassin acetate in *Pseudoplexaura porosa,* 334.
Loligo pealei, survival, unfed, in an aquarium, 389.
Loligo vulgaris, jet propulsion of, 245.
Lumbricus terrestris L., osmotic and ionic regulation in, 247.
Lynn, W. G. See K. W. Chiu, 129.
Lytechinus pictus, division times of whole eggs and haploid and diploid half-eggs of, 184.

M

McMahon, J. J. See W. C. Summers, 389.
Mangum, D. C. Burrowing behavior of the sea anemone *Phyllactis,* 316.
Manzi, J. J. Combined effects of salinity and temperature on the feeding, reproductive and survival rates of *Eupleura caudata* (Say) and *Urosalpinx cinerea* (Say) (Prosobranchia: Muricidae), 35.
Marine cercariae of Woods Hole Massachusetts, 66.
Marine Hydrozoa, hydroid and young medusa stages of *Dipurena,* 109.

Marine trematodes of the Woods Hole area, 66.
Mating in Chlamydomonads, 88.
Maturation of *Hydroides dianthus*, 306.
Medusa, young, and hydroid stages of *Dipurena strangulata*, 109.
Metabolism and distribution of α-ecdysone in pupae of *Antheraea polyphemus*, 115.
Metamorphosis of *Drosophila* discs, 47.
Microtubules of *Stylochus zebra*, 219.
Mitosis of sea urchin eggs, 184.
Morphogenetic fields of *Tubularia*, 344.
Mucopolysaccharides and gut mucous cells of Echinoidea, 286.
Muricidae, environmental effects on, 35.
Muscle, *Limulus*, nitrogenous constituents in, 157.
Mussels, marine, chromosome number, structure and autosomal polymorphism in, 1.
Mytilus californianus, and *M. edulis*, chromosome number, structure and autosomal polymorphism in, 1.

N

Negative staining of microtubules, 219.
Neoconocephalus robustus, body temperature and singing in, 272.
Nephridial clearance in osmotic and ionic regulation in the earthworm, 247.
Nitrogenous constituents of muscle in *Limulus*, 157.
Nuclear-cytoplasmic relations in the mitosis of sea urchin eggs, 184.

O

Ohtaki, T. and C. M. Williams. Inactivation of α-ecdysone and cyasterone by larvae of the fleshfly, *Sarcophaga peregrina*, and pupae of the silkworm, *Samia cynthia*, 326.
Oligochaeta, response to oxygen exposure of, 235.
Osmoregulation in isopods, 96.
Osmotic and ionic regulation in *Limulus polyphemus*, 157.
Osmotic regulation in *Lumbricus terrestris* L., 247.
Oxygen toxicity in annelids, 235.

P

Palaemonetes vulgaris, control by pigment-dispersing and pigment-concentrating substances of the leucophores in, 26.
Papastephanou, C. See J. R. Rice, 334.
Parasitic trematodes, marine larval, of the Woods Hole area, 66.
Partition of cells between blood and tunic in *Halocynthia aurantium*, 354.
Phagocytosis in ascidians, 354.
Phosphotungstic acid, use of in negative staining of microtubules, 219.
Photoperiodism in *Dugesia tigrina*, 77.
Phyllactis, burrowing behavior of, 316.
Pigment-concentrating substances, control of leucophores in the prawn by, 26.
Pigment-dispersing substances, control of leucophores in the prawn by, 26.
Planarians, role of illumination and temperature in the control of sexual reproduction in, 77.
Platyhelminthes, negative staining of sperm microtubules of, 219.
Polymorphism, autosomal, in *Mytilus*, 1.
Population genetics of marine species of the phylum Ectoprocta, 138.
Postlethwait, J. H. and H. A. Schneiderman. Induction of metamorphosis by ecdysone analogues: *Drosophila* imaginal discs cultured *in vivo*, 47.
Prawn, *Palaemonetes vulgaris*, control by pigment-dispersing and pigment-concentrating substances of the leucophores in, 26.
Protein-polysaccharide complexes in tunic, 379.
Pseudoplexaura porosa, isolation, localization and biosynthesis of crassin acetate in, 334.

R

Recovery from hyperbaric oxygen exposure by annelids, 235.
Rectal fluid composition in the earthworm, 247.
Regeneration in ligated stems of *Tubularia* in an electric field, 344.
Regeneration in *Tubularia*, effect of cesium-137 gamma rays on, 194.
Repair after oxygen damage in annelids, 235.
Reproduction of *Hydroides dianthus*, 306.
Reproductive cycle of *Amphiura chiajei* Forbes, 262.
Reproductive rates of marine prosobranch gastropods, effects of salinity and temperature on, 35.
Rice, J. R., C. Papastephanou and D. G. Anderson. Isolation, localization and biosynthesis of crassin acetate in *Pseudoplexaura porosa* (Houttuyn), 334.
Robertson, J. D. Osmotic and ionic regulation in the horseshoe crab *Limulus polyphemus* (Linnaeus), 157.
Rose, F. C. See M. Slaughter, 194.
Rose, S. M. Restoration of regenerative ability in ligated stems of *Tubularia* in an electric field, 344.
Rustad, L. C. See R. C. Rustad, 184.
Rustad, R. C., S. Yuyama and L. C. Rustad. Nuclear-cytoplasmic relations in the

mitosis of sea urchin eggs. II. The division times of whole eggs and haploid and diploid half-eggs, 184.

S

Salinity and temperature, effects on feeding, reproductive and survival rates of marine prosobranch gastropods, 35.

Salinity and temperature, effects on the maturation of *Hydroides dianthus*, 306.

Salinity effects on serum and muscle composition of *Limulus*, 157.

Salt depletion in the earthworm, 247.

Samia cynthia pupae, inactivation of α-ecdysone and cyasterone by, 326.

Sarcophaga peregrina larvae, inactivation of α-ecdysone and cyasterone, 326.

SASTRY, A. N. Reproductive physiological variation in latitudinally separated populations of the bay scallop, *Aequipecten irradians*, Lamarck, 56.

Scallop, bay, reproductive physiological variation in latitudinally separated populations of, 56.

Schizoporella unicornis, genetics of, 138.

SCHNEIDERMAN, H. A. See J. H. POSTLETHWAIT, 47.

SCHOPF, T. J. M. See J. L. GOOCH, 138.

Sea urchin eggs, division times of, 184.

Seasonal cycle of the planktonic larve of *Amphiura chiajei*, 262.

Sephadex LH-20 with gel filtration to separate the dispersing and concentrating substances of the white pigment of the prawn, 26.

Serum, blood, in *Limulus*, inorganic composition of, 157.

Sexual reproduction in *Dugesia tigrina*, role of illumination and temperature in the control of, 77.

SHOEMAKER, D. W. See L. WIESE, 88.

Silkworm, distribution and metabolism of α-ecdysone in pupae of, 115.

Silkworm, inactivation of α-ecdysone and cyasterone by, 326.

Singing in the katydid, 272.

Skin histology in lizard, effects of ACTH on, 129.

SLAUGHTER, M., F. C. ROSE AND A. LIUZZI. The effect of cesium-137 gamma rays on regeneration in *Tubularia*, 194.

Sloughing frequency of normal and thyroidectomized *Anolis carolinensis*, effect of ACTH on, 129.

SMITH, M. J. The blood cells and tunic of the ascidian *Halocynthia aurantium* (Pallas). I. Hematology, tunic morphology, and partition of cells between blood and tunic, 354.

SMITH, M. J. The blood cells and tunic of the ascidian *Halocynthia aurantium* (Pallas). II. Histochemistry of the blood cells and tunic, 379.

Sodium transport in the osmotic and ionic regulation in the earthworm, 247.

SPARKS, A. K. See M. AHMED, 1.

Spermatozoa of *Stylochus zebra*, transitions between helical and protofibrillar configurations in doublet and singlet microtubules in, 219.

Squid, jet propulsion of, 245.

Squid, unfed, survival in an aquarium of, 389.

Starfish, translocation and utilization of amino acids by, 14.

STEINBACH, A. B. Diurnal movements and discharge characteristics of electric gymnotid fishes in the Rio Negro, Brazil, 200.

STUNKARD, H. W. The marine cercariae of the Woods Hole Massachusetts region, 66.

Stylochus zebra, microtubules of, 219.

Substructure of microtubules, 219.

SUMMERS, W. C. AND J. J. MCMAHON. Survival of unfed squid, *Loligo pealei*, in an aquarium, 389.

Survival rates of marine prosobranch gastropods, effects of salinity and temperature on, 35.

T

TAYLOR, P. B. Observations on the function of the frontolateral horns and horn glands of barnacle nauplii (Cirripedia), 211.

Temperature, body, in the katydid, 272.

Temperature and illumination in the control of sexual reproduction of *Dugesia tigrina*, 77.

Temperature and salinity, effects of on maturation of *Hydroides dianthus*, 306.

Temperature and salinity, effects on feeding, reproductive and survival rates of marine prosobranch gastropods, 35.

THOMAS, M. B. Transitions between helical and protofibrillar configurations in doublet and singlet microtubules in spermatozoa of *Stylochus zebra* (Turbellaria, Polycladida), 219.

Tissue defense in ascidians, 354.

Translocation and utilization of amino acids by starfish, 14.

Trematodes, marine larval, of the Woods Hole area, 66.

Tubifex tubifex, response to oxygen exposure, 235.

Tubularia, effect of cesium-137 gamma rays on regeneration in, 194.

Tubularia in an electric field, regenerative ability in ligated stems of, 344.
Tunic and blood cells of ascidians, 354, 379.

U

Ultrastructure, of spermatozoa of *Stylochus zebra*, 219.
Urosalpinx cinerea, effects of salinity and temperature on feeding, reproductive and survival rates of, 35.
Utilization and translocation of amino acids by starfish, 14.

V

Variation in geographically separated populations of the bay scallop, 56.
Venomous organs (supposed) of barnacle nauplii, 211.
VOWINCKEL, C. The role of illumination and temperature in the control of sexual reproduction in the planarian *Dugesia tigrina* (Girard), 77.

W

WALKER, J. G. Oxygen poisoning in the annelid *Tubifex tubifex*. I. Response to oxygen exposure, 235.
Weight changes in different salinities in *Limulus*, 157.
White-pigment–concentrating substance in *Palaemonetes vulgaris*, 26.
White-pigment–dispersing substance in *Palaemonetes vulgaris*, 26.
Whole eggs of sea urchins, division times of, 184.
WIESE, L. AND D. W. SHOEMAKER. On sexual agglutination and mating-type substances (gamones) in isogamous heterothallic chlamydomonads. II. The effect of concanavalin A upon the mating-type reaction, 88.
WILLIAMS, C. M. See T. OHTAKI, 326.
WILSON, W. J. Osmoregulatory capabilities in isopods: *Ligia occidentalis* and *Ligia pallasii*, 96.
Woods Hole, Massachusetts, marine cercariae of, 66.

Y

YUYAMA, S. See R. C. RUSTAD, 184.

Volume 138

Number 1

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

John M. Anderson, Cornell University
Arthur L. Colwin, Queens College, New York
Donald P. Costello, University of North Carolina
Frank M. Fisher, Jr., Rice University
Catherine Henley, University of North Carolina
Meredith L. Jones, Smithsonian Institute
Robert K. Josephson, Case Western Reserve University
Charles B. Metz, University of Miami
Leonard Nelson, Medical College of Ohio at Toledo
Howard A. Schneiderman, University of California at Irvine
Stephen A. Wainwright, Duke University
Carroll M. Williams, Harvard University

W. D. RUSSELL-HUNTER, Syracuse University
Managing Editor

Marine Biological Laboratory
LIBRARY
MAR 19 1970
WOODS HOLE, MASS.

FEBRUARY, 1970

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $5.00. Subscription per volume (three issues), $14.00, (this is $28.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between May 23 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

Copyright © 1970, by the Marine Biological Laboratory

Second-class-postage paid at Lancaster, Pa.

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers, preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

MBL/WHOI LIBRARY
WH 1B1A T